SELECTION AND MUTAGENESIS IN CULTURED AFRICAN VIOLET PLANTLETS

JOHNNY WARBURTON BSc, MSc

This thesis is submitted to the Council for National Academic Awards in partial fulfilment of the requirements for the degree of Doctor of Philosophy

This work was carried out in the Department of Biology, North East London Polytechnic and Department of Life Sciences, Trent Polytechnic, Nottingham in collaboration with Double H House Plants (Ltd), New Milton, Hants.

March 1986

ACKNOWLEDGEMENTS

I would like to thank my Director of Studies, Professor K C Short for his guidance and help throughout this study. I am also indebted to my second supervisors Dr B W W Grout and Dr M A Weatherhead.

The author also acknowledges the support and advice provided by Mr Helmut Gimmler of Double H House Plants.

DECLARATIONS

During the research programme and whilst registered for this degree, the candidate has not been registered for any other award of the CNAA or of any other body.

The material contained in this thesis has not been submitted for any other academic award and represents the individual work of the author.

Johnny Warburton

Supervisors

Professor K C Short

Dr B W W Grout

March 1986

Abstract

In vitro selection of African violet plantlets for tolerance to low temperatures has been investigated. The commercially important Blue Rhapsody variety was used for anther culture studies and an analysis of pollen ontogeny and dimorphism made. It was possible to correlate the stage of pollen development with flower bud diameter and the optimum stage for anther culture studies determined. Futhermore, recommendations are made for the maintenance of donor plants for use in anther culture studies.

The influence of pretreatments of flower buds and a wide range of culture factors were studied in the establishment of a suitable medium for the production of a highly morphogenic callus. This produced abundant numbers of plantlets. Detailed histological and electron microscopic studies revealed that all of the anther derived plants are of somatic origin. Cytological analysis revealed that all plants are either diploid or aneuploid. The majority of plants, when subjected to detailed morphological analysis were found to be similar to the parent. However, three were smaller and were derived from the same anther. It is possible that these plants were of haploid orgin but underwent spontaneous chromosome doubling during their development.

Screening of anther-derived plants, the parent, and a commercially available cold-tolerant variety (Endurance) to grow at a range of temperatures (5-25^{o}C) was studied. This revealed that anther-derived plants grew and produced plants of marketable quality at 15^{o}C. At this temperature the parent showed considerable chlorosis and a marked reduction in growth. Anther-derived plants were not able to withstand temperatures of 10^{o}C as well as the Endurance plants.

An examination of the leakage of electrolytes from tissue subject to low temperatures confirmed that anther-derived plants contain cell membranes which were more tolerant to low temperatures than the parent.

Anther-derived plants which are designated as low temperature tolerant have been subject to three separate screenings both in vivo and in vitro and on this basis are considered to be physiological mutants and not epigenetic variants.

The mutagen EMS, did not significantly increase the number of plants able to survive the screening temperature of 10^{o}C. Attempts to improve the tolerance of plants to low temperature by extending their photoperiod were not successful.

CONTENTS

Chapter Four - Analysis and Evaluation of Anther-Derived African Violet Plants

Chapter Five - Effects of Temperature and Photoperiod on Growth of African Violets

Chapter Six - In vitro Selection for Cold Tolerant Cell Lines of Saint Paulia Ionatha

CHAPTER ONE

INTRODUCTION

1.0 INTRODUCTION

1.1 Brief History of African Violets

In 1892 African Violets were discovered in the East Usambara Mountains of Tanzania by a German civil servant Baron von Saint Paul-Illaire. Saint Paul-Illaire sent seeds back to Germany where they were cultivated and later classified as Saintpaulia ionantha by Herman Wendland, Director of the Royal Botanic Gardens (Hanover) (Wendland, 1893).

Within a year of their discovery African violets gained widespread popularity in horitcultural circles and by 1894, seeds and plants were stocked by continental nurserymen. Although in the same year two plants were imported by a nursery in New York, African violets made little impact on the American ornamental market. However, in the 1920's the Los Angeles firm of Armacost and Royston marketed a quantity of Saintapulias derived from leaf cuttings obtained from a local private estate. In 1927 the nursery imported African violet seeds and raised approximately 1000 plants. Using these plants the firm carried out a programme of cross pollination and selection creating many new hybrids. By 1928 the company had more than half an acre of African violets in cultivation under glass and by 1933 was selling thousands of plants a year. By the late thirties, the plant had become highly popular, and the great vogue for its culture was well under way. Since 1927 the number of species commercially available has increased from 2 to 27 and in less than 100 years this attractive plant has become a favourite throughout the world.

1.2 Commercial Importance of African Violets

World trade in the African violet is worth an estimated $30 million per year. Over the past ten years this plant

has maintained a position (based on total sales) in the top fifteen most popular indoor ornamentals in both North America and Europe (Holdgate, 1977; Anon, 1980). Thomas Rochford & Sons, Ltd, Britains largest producer of indoor ornamentals, grow over three-quarters of a million Saintpaulias annually. This quantity represents only a small fraction of the total number produced annually in the UK.

Due to its regenerative potential, Saintpualia has been used as a model plant for assessing; mass micro-propagation techniques for commercial use (EEC - Cost 87 Project); the potential of haploid plants in crop improvement programmes (Grout and Weatherhead, 1980) and the uses of mutagenic agents in plant breeding (Broertjes, 1972).

1.3 Vegetative Propagation

African violet is traditionally propagated vegetatively from leaf cuttings. Commercial propagation of Saintpaulia is usually carried out by placing cuttings in seed trays containing a 1:1 mixture of sand and vermiculite or sand and perlite. The seed trays are placed on a heated bench ($20^{o}C$) and covered with plastic to maintain high humidity. Between 13-16 weeks, 2-8 daughter plants are obtained from each cutting.

However, cuttings and plants of African violet cultivated under greenhouse conditions are very susceptible to stem and root rots caused by destructive phytopathogens such as; Phytophthora sp (Ford, 1953; Plate and Krober, 1972), Erwinia chrysanthemi (Knauss and Miller, 1974) and Pythium sp (Ford, 1953) leading to a marked reduction in the efficiency of propagation. Thus the combination of the low number of plants produced by each leaf cutting coupled with losses due to disease and the steadily increasing cost of heating glasshouses is making the commercial

propagation of Saintpaulia an extremely expensive, risky business. It is highly understandable therefore, that many commercial growers are seriously considering tissue culture techniques as a means of rapid clonal propagation.

1.4 Tissue Culture of African Violet

The first report of morphogenesis in tissue culture of African violet was published by Kukulczanka and Suszynska (1972). The above workers, described the induction of adventitious shoot buds from isolated leaves cultured *in vitro* leaves. They also noted that such buds arose from either single epidermal cells or from epidermal cell groups. This work was later confirmed by Start and Cumming (1976) using leaf pieces instead of whole leaves to regenerate shoot buds. Maximum shoot induction occured on modified MS medium containing $0.1mgl^{-1}$ NAA, $5.0mgl^{-1}$ BAP and $125mgl^{-1}$ adenine sulphate. Start and Cumming concluded that a single cultured leaf of African violet could produce approximately 500 plants.

Work by Kukulczanka and Suszynska (1972) demonstrated that petioles separated from the leaves have no regenerative properties and undergo complete necrosis on MS medium. However, Flores et al (1976) reported the microculture of leaf pieces and epidermal strips on B5 medium supplemented with various concentrations of NAA and BAP. In the same year Grunewaldt described the successful regeneration of plants from callus derived from petiole epidermal cells. Furthermore, Bilkey et al (1978) showed that 5,000 commercially useable plantlets could be generated from a single petiole in 3-4 months. Finally, Harney and Knap (1979) produced normal African violet plants directly from petiole cross sections without an intermediate callus phase using MS medium supplemented with $0.5mgl^{-1}$ NAA and $0.5mgl^{-1}$ kinetin.

Recenly, Bilkey and Cocking (1981) demonstrated the differential regeneration capacities of epidermal and sub-epidermal tissues of African violet. They found that plants derived from sub-epidermal tissues were more vigorous than their epidermal counterparts.

In 1977 Vazquez et al demonstrated the morphogenetic potential of *in vitro* cultured excised floral parts of African violet. They discovered that precocious adventitious shoot production occured directly from the surface of ovary, sepal and petal tissues cultured on MS medium containing $1mgl^{-1}$BAP and $1mgl^{-1}$NAA. Subsequent work by Vazquez and Short (1978) showed that in cultured petal tissue, shoots developed from small green nodular areas which were formed in the epidermis but did not appear to be related to particular cells or loci within the epidermal layer.

Sitbon (1973) reported the production of plantlets regenerated from anther callus. However, the origin and ploidy level of both callus and plantlets were not given. Hughes et al (1975) working in the USA were the first to describe the production of haploid plantlets directly from cultured anthers. This work was later confirmed by another American research group (Smith, et al, 1981) and by workers in the United Kingdom (Weatherhead, et al, 1982). The American groups have reported an anther response ranging from between 3.6-30% with 1-5 plants produced per anther. However the British group showed that between 9 and 31% of anthers could produce callus and that 50-200 plants could be regenerated per anther.

In 1977, Hughes reported that protoplasts could be obtained from diploid and haploid plants grown in axenic culture but not from greenhouse grown plants. She investigated the various factors affecting the yield

of protoplasts from leaf and petiole tissue and found that the most effective combination of enzymes was 2% cellulysin, 0.25% Macerozyme, 0.25% hemicellulose and 0.25% pectinase. Furthermore, a combination of 0.27M sorbitol and 0.27M mannitol gave the most consistent protoplast yields. However, as of yet no plants have been regenerated from African violet protoplasts. Five years later Bilkey and Cocking (1982) described the isolation of protoplasts from Saintpaulia petiole callus using a non enzymatic method. Thin walled callus was produced from petiole cross-sections on a solid medium containing high levels of auxin (2,4-D). Protoplasts were released by slight teasing of the callus tissue with dissecting needles. These non-enzymatically isolated protoplasts provide a suitable experimental system for various physiological studies including assessment of the toxicity of cell wall degrading enzymes.

Bilkey, Davey and Cocking (1982) reported that by rupturing auxin induced, highly vacoulate, thin-walled callus cells of Saintpaulia, large numbers of enucleate subcellular units (microplasts) were released. These enucleate microplasts are surrounded by an inner membrane of the cell, most probably derived from the tonoplast. When microplasts were isolated from Saintpaulia callus and cultured on a medium supplemented with growth substances they formed a thin wall and underwent budding. The above authors suggest that microplasts could be useful for plant genetic manipulations.

1.5 Conventional Versus Tissue Culture Methods

Once it was shown that tissue culture techniques could be used for propogation of African violets many research groups produced schemes for mass propagation of Saintpaulias from either leaves (leaf sections); (Start and Cumming, 1976; Cooke, 1977; Moncousin, 1978; Jacob, et al, 1980; Takayama and Misawa, 1982) or leaves

and petiole cross-sections (Le and Collett, 1981;Lim-Ho and Lee, 1982; Xu, 1984).

However, although using micropropagation techniques there is the advantage of producing uniform plants at very high propagation rates (Cassells and Plunkett, 1984) compared to conventional procedures it is generally a labour intensive method requiring specially trained personnel. Therefore, its introduction into nurseries is restricted by economical factors. In a recent report, a comparison of; mutliplication rate, required hours and square meters per day needed to produce 10,000 African violet plants, was made between tissue culture and conventional propagation methods (Theiler-Hedrich and Theiler-Hedrich, 1983). These workers concluded that although the multiplication rate and square meters per day were far superior, tissue culture methods, were ten times more labour intensive than conventional propagation methods. This indicates that for African violet tissue culture methods are still in competition with traditional propagation methods.

At present improvements to the tissue culture procedure of African violets are being undertaken by the Cost 87 Project funded by the EEC.

1.6 ANTHER AND POLLEN CULTURE

1.6.1. Anther Culture

The first haploid tissue cultures were initiated by the divisions of the vegetative cell in the mature pollen grains of *Ginkgo biloba* (Tulecke, 1953, 1957) Haploid tissue cultures were also obtained from the pollen of *Taxus* by C D Larue of the University of Michigan (Tulecke, 1959), *Torreya nucifera* (Tulecke and Sehgal, 1963) and *Ephedra foliata* (Konar, 1963).

However, no shoot or plantlet formation took place in any of these cultures.

All of the early attempts to obtain haploid tissue cultures from pollen grains in cultured anthers of angiosperms failed, although callus tissues of somatic origin were often formed from the filament or the connective regions of the anther. Some of the species in which profileration of the somatic tissues of the anther has been reported are Datura innoxia (Guha and Maheshwar 1967), Chrysanthemum (Watanabe et al, 1972), Triticum aestivum (Shimada and Makino, 1975) and Brassica napus (Thomas and Wenzel, 1975b).

Yamada et al (1963) were the first to report the isolation of a haploid angiosperm tissue from anther cultures of Tradescantia reflexa; they believed that the tissue developed from microspore mother cells. Soon after this, Guha and Maheshwari (1964) observed the formation of embryo-like structres developing from culturered Datura innoxia anthers and subsequently confirmed the haploid nature of these embryoids and their origin from immature pollen grains (Guha and Maheshwari, 1966).

The above workers cultured Datara innoxia anthers on agar-gelled media. This media contained salts and sugar according to the well-known formulations of plant tissue culture media, namely those of Nitsch and White along with certain hormones or growth supplements such as fruit juices or coconut milk. The above technique served well for the majority of species under investigation. However, many species have proved to be toally recalcitrant.

Thereafter, in an effort to elicit a response from such species, as well as to enhance the response

in general, several workers have sought to modify the original procedure of Guha and Maheshwari, Notably, Wernicke and Kohlenbach (1975, 1976) found that culture of anthers in liquid, instead of agar-nutrient media resulted in higher yields of pollen embryos.

Since the early pioneer work of Guha and Maheshwari (1964, 1966) androgenesis has been recorded in 171 speices belonging to some 60 genera and 26 families of angiosperms (Maheshwari et al, 1982). This research has included work on economically-important crops such as Brassica oleracea (Kameya and Hinata, 1970), Brassica napus (Thomas and Wenzel, 1975b), Camellia sinensis (Iyer and Raina, 1972), Cocos nucifera (Thanh-Tuyen and Guzman, 1983) Coffea arabica (Sharpe et al, 1973, Hevea brasiliensis (Chen et al, 1982), Hordeum vulgare (Clapham 1971, 1973; Cossette and Pause, 1983), Oryza sativa (Niikeki and Oono, 1968, 1971; Chen, 1976, 1977 and Schaeffer et al, 1985), Saccharum spontaneum (Chen et al, 1979, Fitch and Moore, 1983, 1984), Secale cereale (Wenzel and Thomas, 1974; and Friedt et al 1983), Triticum aestivum (Ouyang et al, 1973; Shimada and Makino, 1975), Triticale (Wang, et al, 1973), and Zea mays (Inst of Genetics, 1975; Jialin, et al, 1983). Successful antner culture has been carried out on a number of Solanaceous crops namely Solanum tuberosum (Dunwell and Sunderland, 1973), tomato (Sharpe, Dougall and Paddock, 1971, Krueger-Lebus, et al, 1983), Capsicum (George and Narayanaswamy 1973; Wang, et al, 1973b) and the eggplant (Raina and Iyer, 1973). Progress has also been made on members of the Leguminosae, particularly Pisum sativum (Gupta, 1976), Trifalicum alexandrinum (Mokhtarzadeh and Constantin, 1978), Arachis hypogaea(Bajaj, et al, 1981) and Cassia siamea (Gharyal, et al, 1983).

Information on anther culture of ornamental plants is unfortunately limited. Low frequency of production of n, 2n, 3n and 4n plants has been reported in Petunia (Raquin and Pilet, 1972; Engvild, 1973; Wagner and Hess, 1974; Sangwan and Norreel, 1975; Gupta, 1982; Raquin, 1982) but this appears to be the only ornamental genus to have attracted attention in a family containing many such plants This may be because the ornamental species have been found to be less responsive to anther culture than their more amenable relatives. Apart from the Solanaceous ornamental's low frequencies of induction have been reported in Begonia (Khoder et al, 1984), Lilium longiflorum (Sharp, et al, 1972b) and Pelargonium hortorum (Abo El-Nil and Hildebrandt, 1973;Bennici, 1974). Also this technique has been extended to the genera of the Ranunculaceae Anemone (Sunderland and Dunwell, Johansson, et al, 1982; Johansson, 1983; Johansson and Eriksson, 1984), Paeonia (Sunderland, 1974; Sunderland and Dunwell, 1974;Zenkteler, et al, 1975; Ono and Harashima, 1981, 1983), Helleborus (Zenkteler, et al, 1975) and one member of both the Commelinaceae Tradescantia bracteata (Sunderland, 1977) and the Gesneriaceae, Saintpaulia ionantha (Hughes, et al, 1975;Smith, et al, 1981, Norris, et al, 1982; Weatherhead, et al, 1982; Bhaskaran, et al, 1983)

1.6.2. Pollen Culture

Many workers have attempted the induction of haploids by culturing isolated pollen because of many potential advantages. Primarily, by the use of pollen culture, one can eliminate the possibility of contaminating profilerations of plantlets from the anther wall. Secondly, overcrowding of pollen grains can be eliminated and so also any deleterious influence of possible inhibitors from the anther wall.

Finally, the regulation of androgenesis can be studied in pollen culture with a much higher degree of precision than can be realised with anther culture. So much so that pollen grains could be plated and handled like microbes.

The earliest studies on the culture of isolated pollen grains were made by Nitsch and Norreel (1973) on Datura innoxia and Petunia hybrida. However, in the hand of other workers the results were often unreliable and inefficient (Sunderland and Roberts, 1977). Successful attempts therefore were made to modify the technique by taking pollen grains from anthers precultured for a few days. These included anthers of Nicotiana tabacum (Wernicke and Kohlenbach, 1977), Solanum tuberosum (Weatherhead and Henshaw, 1979) and Oryza sativa (Chen, et al, 1980). The logic behind preculturing is that the critical phase for initiation of androgenesis is passed in the normal manner within the whole anther and the pollen grains are then more amenable to culture.

Later work on rye by Wenzel, et al (1975) and on Nicotiana tabacum by Wernicke, et al (1978) reflect a further refinement of the technique described above. In both cases, an enriched fraction of potentially embryogenic grains were separated from the pollen isolated from precultured anthers. This was achieved by layering the pollen suspension over highly concentrated sucrose solution followed by density gradient centrifugation or by density gradient centrifugation employing sucrose with Percall.

To bypass the deleterious effects of isolating pollen by mechanical means, Sunderland and Roberts (1977) proposed a new technique of isolating Nicotiana

pollen. The method consists of culturing anthers in liquid medium then transferring them to fresh medium at regular intervals. This method exploits the observation that in a liquid medium anthers dehisce spontaneously and shed pollen which develop into embryoids. This technique has also been shown to work well in Hyoscyamus niger (Sunderland and Wildon, 1979) as well as in Datura innoxia (Tyagi, et al, 1979).

Further more the use of whole anthers, callus tissues or leaves as nurse tissue to induce androgenesis has been successfully demonstrated in Lycopersicon esculentum (Sharp and Raskin, 1972) and Hordeum vulgare (Zenkteler and Stefaniak, 1982). In the former case only clumps of green parenchyma-like cells were observed after one month of culture, whereas in barley, haploids were regenerated from the multicellular microspores.

Several investigations habe been undertaken in an effort to establish ab-initio pollen culture. Heberle-Bors and Reinert (1980) working with Nicotiana-tabacum var. Badisher Burley separated embryogenic pollen from non-embryogenic pollen by centrifugation, from unprecultured anthers, using Percoll with sucrose. However the number of embryos formed after 6 weeks was a rather low frequency of 0.002 - 0.006%. Rashid and Reinert (1980, 1981a) have been able to increase the frequency of embryo formation to between 2 and 5% by giving prior cold treatment to flower buds and by modifying other procedures, namely: 1) use of lower concentration of sucrose in the pollen separation medium; 2) omission of asparagine and glutamine from the culture medium; 3) raising the pH of the medium to 6.8. In the same year the above workers reported an elevated frequency of embryo formation by pollen, in the separated embryogenic fraction, to as much as 27% when flower buds of a slightly more advanced stage were selected for cold treatment and donor plants were maintained under

short days at 15°C just before excision of buds.

Recently, attempts have been made to obtain successful induction of embryogenic divisions from pollen grains isolated directly from anthers which have not been subjected to a cold treatment or preculture. In 1981 Ono and Harashima reported divisions in pollen cultures of peony without any prior selection of embryogenic grains. The following year, the same workers obtained plantlet formation from pollen grains isolated directly from tobacco anthers which were not subjected to a cold treatment or precultured. Pollen was cultured initially for 5 to 7 days in distilled water followed by transfer to a mineral salt medium containing sucrose and glutamine. In 1983 Nagmani and Raghavan reported the induction of embryogenic divisions in isolated pollen grains of Hyoscyamus niger after 7-10 days using a single-step method. In this instance pollen was cultured on filter paper discs (contained in sealed Petri dishes) impregnated with Bourgin and Nitsch's (1969) liquid basal medium supplemented with $3mgl^{-1}$ Kinetin or $2mgl^{-1}$ 2,4-D and $5mgl^{-1}$ Benzylaminopurine.

In particular context with pollen culture it is important to note that in certain plants a dimorphism is seen in pollen grains, even in anthers maturing in vivo. In Hordeum vulgare cv. Sabarlis (Sunderland 1974) and cv. Akka (Dale, 1975), Nicotiana tabacum (Horner and Street, 1978) and Paeonia hybrida (Sunderland, 1974) there are two populations of microspores. Some are large and stain densely in acetocarmine whilst others are smaller and stain lightly. It is the latter type which gives rise to pollen embryoids. Indeed, workers were quick to exploit this phenomenon. Wernicke, et al (1978), Herberle-Bors and Reinert (1980) and Rashid and Reinert (1980, 1981a,b) have separated highly embryogenic fractions by density centrifugation

in N.tabacum and dimorphism has proved to be an enormous advantage in these studies.

In summation it is important to emphasise that the frequency of these embryogenic pollen grains is determined before culture by genotype and environment of the donor plants (Heberle-Bors, 1982, 1984). For the donor plants, they are functionally sterile pollen grains and as a rule, their frequency is dependent upon factorsaffecting male sterility and sex balance of the flowers (Herberle-Bors, 1982). In tobacco (Herberle-Bors, 1983) treatment of the pollen donor plants with feminizing agents (such as Alar 85) strongly increased embryogenic pollen grain formation and pollen plant production. In wheat, cytoplasmic male sterile lines have bene found producing pollen which consists almost exclusively of embryogenic pollen grains (Herberle-Bors and Odenbach, 1984).

However, this new emphasis on the role of the genotype and environment of the parent plant should not underestimate the nutritional requirements for successful pollen culture.

1.6.3. Staging of Anthers

The formation of a tetrad of haploid microspores (enclosed within a rigid callosic wall, at the end of meiosis) marks the beginning of the male gametophytic phase in flowering plants. The callose wall is soon broken down by the release of (β-1, 3-glucanase) from the surrounding tapetal tissues, and the microspores are released into the anther locule. Thereafter the microspore nucleus, with accompanying important cytoplasmic changes, divides initially to form two unequal cells, a large vegetative cell and a small generative cells.The latter undergoes one final mitotic

division, either within the anther or after anther dehishence and pollen germination in the tissues of the stigma/style, to give rise to two male haploid cells. (Vasil, 1973a, 1974).

One of the most critical requirements for the successful induction of androgenesis in-vitro is the stage of microspores at the time of anther excision and culture. Experience has shown that in most species maximum response is obtained when anthers are excised just before, during, or immediately following the mitosis of the microspore nucleus. This is perhaps because pollen grains are then in a sense uncommitted to either the androgenic or their normal mode of development. Sunderland (1974) defined six anther stages by grouping anthers into an age sequence on the basis of cytological events. The six stages are described as follows Stage 1 - Anthers containing spore tetrads or spores just released from the mother cell. Stage 2 - Anthers containing vacuolate spores in which the nucleus is situated close to the spore wall. Stage 3 - Anthers containing spores in the process of, or after DNA replication. This stage is distinguished from Stage 2 by the difference in the size of the nucleus. Stage 4 - Anthers containing spores undergoing the first pollen division. Stage 5 - Anthers containing young pollen grains in which the two presumptive gametophytic cells are separated by a curved wall. The vacuole carried over from the microspore is still present. Stage 6 - Anthers contianing young pollen grains in which the vacuole is resorbed and the vegetative cell is filled with cytoplasm. The generative cell is detached from the intine and in the tricellular group may have divided again. Starch deposition is minimal.

In a highly responsive species like Nicatiana tabacum a peak response is obtained at stages 3 to 5, that is, just before, during or just after the

first pollen division (Dunwell, 1976). However, in Brassica oleracea (Kameya and Hinata, 1970) and in Nicotiana knightiana and Nicotiana sylvestris (Primo-Millo and Sunderland, 1976; Sunderland and Dunwell, 1977) anthers excised at the two-celled young pollen grain stage were most productive (Stages 5 to 6). In such cereals as Hordeum vulgare (Clapham, 1971), Triticum aestivum (Wang, et al, 1973), and Oryza sativa (Wang, San and Chu, 1974) young, stage 3 microspores gave the peak response. Finally, in Arabidopsis thaliana, Lycopersicum esculentum, and Vitis vinifera, anthers cultured at various stages of meiosis also gave rise to haploids although the best results were obtained from anthers containing tetrads or microspores just released from the tetrad callose wall (Gresshof and Doy, 1972a, b, 1974).

Although some synchroneity is seen during the development of pollen, significant differences are also known to occurin the stages of development within a single anther, or between different anthers of a flower bud (Vasil, 1967). Staging of anthers prior to culture is therefore important and can be achieved by removing one anther from each bud to determine the average stage of pollen development. Such, staging by means of direct cytological examination is time consuming and laborious and in practice it is often convenient to correlate the anther stage with an external morphological character, such as, corolla length (Nitsh & Nitsch, 1969), bud diameter and the length of the emerging flag leaf or spike. However, such correlations tend to break down unless plants are grown under rigidily standardised conditions and are of the same age. (Sunderland, Collins and Dunwell, 1974, Dunwell, 1976).

1.6.4. Factors Influencing Anther and Pollen Culture

1.6.4.1. Donor Plant: Physiological Status and Genotype

One of the most important factors affecting the frequency of haploid induction is the physiological conditon of the donor plant. Plants grown in the field generally prove to be better donors than those raised in the greenhouse or growth chambers. This is largely because requirements for optimal growth are either unknown or not provided under the latter conditions. Seasonal variations in anther response are common in a number of species including Solanum tuberosum (Dunwell and Sunderland, 1973), Hordeum vulgare (Dale and Humphreys, 1974) and Triticum aestivum (Picard and De Buyser, 1975). Temperature, photoperiod and light intensity used for the growth of the donor plants all play important roles in determining anther response (Sunderland and Dunwell. 1977). In Nicotiana tabacum cv. White Burley the highest yields of plantlets were obtained from plants grown under short days and high light intensities (Dunwell, 1976). More recently in N. tabacum cv Badisher Burley a ten fold increase in the number of embryogenic grains was obtained by growing plants under short days and reducing the growing temperature from 24°-18°C (Herberle-Bors and Reinert, 1981; Herberle-Bors, 1982). Keller and Stringham (1978) showed that rape seedplants grown at low temperatures gave the highest embryo yields.

However, in Nicotiana knightiana anther response was doubled by inreasing the donor plant growth temperature from 14°-20°. (Primo-Millo and Sunderland, 1976).

A further factor which strongly affects the response of anthers even from plants grown under favourable

conditions is the age of the donor plant. Anthers from buds formed at the beginning of the flowering period are most suitable, because anther response declines with increasing age of the donor plants.

The various physiological factors described above must affect the endogenous levels of hormones as well as the general nutritional status of the sporogenous and the somatic tissues of the anther. It follows that in anther and pollen culture, reproducible results can be expected only by the use of plants grown under standard controlled environments and by the use of buds from the same age of plant (Sunderland and Dunwell, 1977).

However, certain other treatments to the donor plant have been used to increase yields of plants from anther and microspore cultures. These include the removal of the apical region of the inflorescence (Picard, 1973), application of hormones, growth regulators, feminising agents (such as Alar 85) (Wang, et al, 1974; Bajaj, et al, 1977; Colhoun, et al, 1983; Heberle-Bors, 1983), regular removal of old flower buds (Nitsch, 1975), clipping plants at the ground level and allowing them to stand in water for 1 or 2 days (Wilson, 1977), nitrogen starvation (Sunderland, 1978; Rogozinska and Goska, 1982) addition of mineral nutrients (Herberle-Bors and Reinert, 1979).

The wide variations in androgenic responses among even closely related taxa has focussed attention on the role of the donor plant genotype. Not only the species within a genus but even cultivars of the same species often show markedly dissimilar responses in culture (Nitsch, 1969; De Moraes-Fernandes and Picard, 1983; Marsolais, et al, 1984; Thurling and Chay, 1984; Heberle-Bors, 1984). Also, higher frequencies of anther response are obtained in lines that themselves have been derived from microspores (Picard and De Buyser, 1977).

1.6.4.2. Pretreatment of Flower Buds and Anthers

It has been found that temperature stress pretreatment given to flower buds prior to culture, or in some instances directly to anthers, can markedly enhance the anther response. Nitsch and Norreel (1973) found an increased response of D. innoxia anthers that were taken from excised flower buds stored for 48 hours in a refrigerator (4-7°C) initial observations have been confirmed by several independent studies, and the chilling pretreatment are now used extensively (Tyagi, et al, 1979; Sunderland and Roberts, 1979; Taguchi and Mii, 1982). However, chilling pretreatments are not always necessarily the most effective means of enhancing the production of pollen plants from cultured anthers. Thus for bud pretreatment in tobacco (Sunderland, 1978) and henbane (Sunderland and Wildon, 1979) moderate temperatures (about 15°C) given over periods of up to 21 days.

Sunderland and Dunwell (1977) advised that in the case of species like D. innoxia which have large buds, individual buds at the critical stage should be placed with their stalks in water and kept in a refrigerator at 4-5°C. However, species with small buds are best treated as whole or part inflorescences and grasses as whole tillers. An alternative and less time consuming method is to enclose the excised parts in polythene bags, then wrap in aluminium foil to minimise water loss, prior to incubation. However, Huang and Sunderland (1982) showed that for pretreatment of barley anthers; excised spikes kept in Parafilm-sealed Petri dishes (containing drops of water to maintain humidity), were more effective than excised tillers kept in water and wrapped in polythene.

How such treatments favours formation of haploid embryoids is still open to question. It has been speculated

that the chilling of anthers prior to culture increases the number of microspores that undergo atypical mitosis. This results in the formation of two equal and/or identical cells instead of the distinct characteristic generative and vegetative cells. Alternatively, chilling can delay the senescence of anthers, thereby maintaining a higher number of viable and embryogenic pollen grains. Centirfugation of anthers particularly in combination with cold treatment has also been found to enhance plantlet production. One possible explanation could be that both low temperatures as well as centrifugation extend their effects through dissolution of microtubules which are known to reorganise during division.

In any event, past research has shown that the correct method and optimal temperature required for temperature stress pretreatment has to be determined systemically for each species and cultivar.

Recent work has shown that, a reduced atmospheric pressure for a few hours (Imamura and Harada, 1980) or a water saturated atmosphere for 2 or 3 days (Dunwell, 1981) prior to inoculation of tobacco anthers can also enhance their response.

1.6.4.3. Culture Medium

The nutrient requirements for inducing a switch from the gametophytic phase to adrogenetic development are quite simple. In Nicotiana this can be achieved by placing excised anthers on water-agar or on a simple 2% sucrose solution gelled with 0.8% agar (Nitsch, 1971). In D. innoxia growth of the pollen can be induced by simply floating anthers on a 2% sucrose solution and incubating at 25°C (Sunderland, 1974). However, under such conditions only a small number of embryoids were formed and those did not develop

beyond the globular stage. In both Datura and Nicotiana a simple sucrose and mineral salts medium without any growth substances or vitamins is sufficient to ensure development to the plantlet stage (Nitsch and Nitsch, 1969).

As a basal medium, either the formulation of Murashige and Skoog (1962) (MS) or the modification of it introduced by Bourgin & Nitsch (1967) (H medium- contains the MS major salts at approximately half strength) is suitable for plantlet production in a number of species including Datura innoxia, Nicotiano tabacum, Paeonia hybrida and Hyoscyamus niger. In Atropa belladonna, a fair degree of success was obtained when anthers were cultured on Linsmaier and Skoogs medium (1965) (LS). A high nitrate medium was used in Brassica campestris (Keller, et al, 1975) the formulation being a modification of the B5 medium of Gambourg, et al (1968). Chu, et al (1975) have shown the beneficial effect of an increased concentration of nitrate and a low concentration of ammonium (modification of Miller's 1963 medium) for anther culture of rice thus emphasising the need to identify influential components of nutrient media. For obtaining optimal responses various media must be tested for each species investigated as success may depend largely on the choice of medium and its components.

A carbon source of 2-4% sucrose has been most frequently used (Nitsch, 1969; Hidaka, 1984). However, much higher concentrations , e.g. 6-12% sucrose, have been found beneficial in some cases, especially in cereals (Clapham, 1977). In Brassica campestris (Keller,, et al, 1975) and Solanum tuberosum (Sopory, 1979) it has been recommended that initially anthers should be cultured on high-sucrose media then transferred to low levels of sucrose for optimal post-induction

growth. The requirement for sucrose is specific and cannot be substituted by metabolically inert compounds or by other disaccharides (Keller, et al, 1975), although the possible role of sugars in osmotic regulation has also been pointed out (Reinert and Bajaj, 1977; Sunderland and Dunwell, 1977). However, this view does not seem to be supported by experiments in Solanum tuberosum since sucrose along with mannitol did not match the response obtained by a high level of sucrose (Sopory, 1979).

Recent research has shown that the early stage of pollen embryogenesis, that is the transformation of pollen microspores into embryoids, is inhibited by the presence of chelated Iron. Consequently, many more microspores develop into globular embryoids on media without chelated iron then on that with chelated iron. (Havranek and Vagera, 1979). The presence or absence of iron ions or chelate apparently has no direct correlation with the initiation of androgenesis, however it produces a great regulatory effect on the subsequent development of the embryoids.

Experiments on tobacco and Datura show that for the development of the embryoid into a plant it is necessary to add FeEDTA to the medium. However, it was also demonstrated that the presence of any EDTA in the medium has the same morphoregulatory effect. This was explained by the formation of a complex with traces of iron ions (in distilled water, surcrose or agar) polluting the culture media (Vagera and Havranek, 1982b). Recently, Vagera and Jilek (1984) discovered that the traces of iron present in the agar were sufficient for the development of globular embryoids into a complete plant. The outcome of this discovery was a recommendation from the above workers to decrease the amount of iron and leave the commonly used amount of chelate in the media. In doing so an optimal morpho-regulatory effect in androgenesis will be achieved.

Also the vitality of the tissue culture will be lengthened and ageing as well as necrosis of the anthers will decrease.

Plant growth substances particularly, auxins and cytokinins play an important role in the induction of androgenetic development. A recent survey of the many reports of anther culture by Maheshwani, et al (1982) showed that in a majority (80%) one or another hormone, usually an auxin or a cytokinin, has been a component of the medium.

In cereals particularly, both auxins and cytokinins have to be used. In such species direct embryogenesis is rare and plants have to be differentiated from callus. Therefore, for callus induction cuisin is generally required, whereas for subsequent regeneration and plantlet formation low levels of auxin or the basal medium alone are beneficial.

Even in those species which normally give rise to embryoids directly from the microspores, callus tissues are obtained when auxins and/or cytokinins are added to the nutritent media (Sunderland and Wicks, 1971). Therefore the presence or absence of auxins in the medium, as well as the endogenous levels of auxins in the anther tissues, appears to determine the pathway of haploid formation. This is clearly seen in Hyoscyamus niger (Raghaven, 1978). The elimination of auxin from the medium prevents callus formation and stimulates direct embryoid production.

Furthermore, Sunderland and Dunwell (1977) emphasise the importance of the hormone levels in the media. In Datura innoxia, low hormone levels induce callus formation from the pollen. However if the hormone concentration is elevated, pollen growth is inhibited and callus formation is triggered from either the wall tissues or the cut end of the filament.

Although hormones have been used for pollen cultures of Nicotiana tabacum (Reinert, et al, 1975) and Solanum tuberosum (Weatherhead and Henshaw, 1979), some workers have found that they are not always necessary for embryo formation (Nitsch, 1974b).

The use of complex mixtures of organic origin, such as yeast extract, coconut milk, casein hydrolysate, potato extract and crushed anthers themselves have been found very beneficial in promoting embryogensis. Remarkable success has been obtained by Chinese workers employing potato extract. (Chung, et al, 1978).

Nitsch (1974b) analysed the extracts of embryogenic anthers and emphasised the importance of such amino acids as glutamine and serine. In similar studies, myo-inositoland glutamine have also been shown to ennance the frequency of pollen embryo formation. This is particularly true in the culture of isolated microspores. In Nicotiana embryogenesis was achieved when induced pollen was cultured on media supplemented with glutamine, serine and myo-inositol by Nitsch (1974b) and Sunderland and Roberts (1977). Recently aspargine has been shown to be beneficial for pollen culture of Solanum (Weatherhead and Henshaw, 1979).

Liquid culture media have bene shown to be superior to agar media in some instances (Wilson, et al, 1978). This may result from the presence of inhibitors in the agar. Addition of activated charcoal to agar media has been shown to enhance haploid production (Nakamura and Itagaki, 1973; Anagnostakis, 1974; Genovesi and Collins, 1982; Johansson, 1983). possibly through adsorption of inhibitors present in the agar or the medium or those being released from the senescing anther wall (phenolics). However, it has also been shown that activated charcoal is capable of adsorbing both napthaleneactic acid and several cytokinins

(Weatherhead, et al, 1978). This together with the fact that other useful components inthe media, such as chelates can be absorbed (Herberle-Bors, 1980) makes it important to use activated charcoal with caution.

Polyvinylpyrrolidone (PVP) and Polyvinlypoly-pyrrolidone (PVPP) (an insoluble, high molecular weight, cross-linked form of PVP) have recently been found to promote pollen embryo production in anther culture of Datura (Tyagi, et al, 1981b; Babbar and Gupta, 1982). The effect is possibly due to adsorption of substances (phenolics) that emanate from cultured anthers and inhibit the development of pollen grains into embryos.

Furthermore, incorporation of the antitoxant L-cysteine-HCl into the medium cantaisl pheral oxidation and increases the frequency of pollen plantlet production.

1.6.4.4. Physical Factors

The manner in which anthers are placed on the medium is important. Sopory and Maheshwari (1976a) found that placing the anthers horizontally on the surface of the medium resulted in the formation of the largest number of embryoids. Immersion of even a portion of the anther in the agar reduces anther response.

The number and density of anthers per culture vessel also influences the response of anthers. These parameters may determine the behaviour of the anthers through the nature of the gaseous atmosphere around, as the presence of carbon dioxide and/or ethylene in the culture environment is said to influence the

production of haploids (Wang, et al, 1974). Dunwell (1979) showed that the removal of specific components of the culture atmosphere (ethylene, carbon dioxide, oxygen) influenced the response of the anthers. Johansson (1982) found that by elevating the CO_2 concentration to 2% stimulated embryogenesis in anther cultures of Anemone and Papaver.

Generally, anther and microspore cultures are incubated between 25-30°C, as at extreme temperatures, the response declines sharply. However, in Brassicas Keller and Armstrong have shown that sharp increases in embryoid formation occur when anthers are incubated at 30-35°C. However temperatures lower than 25°C reduce anther response.

Light is not known to be required for the induction of androgenetic development although it is beneficial for post-inductive growth and enhances plantlet yields (Sunderland, 1971). It is now established practice to illuminate cultures after a short inductive period in darkness (Sunderland and Roberts, 1977). Continuous light is generally inhibibory, and isolated microspores appear to be more sensitive to light than are whole anthers (Vasil, 1980). For pollen culture of Nicotiana tabacum, Nitsch (1977) found red light more suitable then blue or low intensity white light. However, Sopory and Maheshwari (1976a) found red light inhibitory.

1.7. Origin of Haploids

Several distinct pathways lead to androgenetic development, and the pattern of development determines the ploidy level of the resulting callus tissue or embryoids. Four principal patterns of haploid formation have been described.

a. Vegetative Cell

The normal course of division follows to form the smaller generative cell and the larger vegetative cell, but the embryo develops from the vegetative cell alone. The generative cell either takes no part in the formation of the haploid tissues or divides two or three times, but it eventually degenerates. The pathway via the vegetative cell (the A pathway) was first shown by Sunderland and Wicks (1969, 1971) in Nicotiana tabacum cv. White Burley. Such development has been studied most extensively and is decribed in several species of Paenia Triticale and T aestivum.

b. Generative Cell

The formation of haploid embryoids by the divisions of the generative cell is not common and has so far been described in Hyoscyamus niger (Raghaven, 1976, 1978). Raghaven discovered that in this species the vegetative cell either does not divide or undergoes a few divisions to form a suspensor-like stucture attached to the embryoid arising from the generative cell.

c. Vegetative and Generative Cell

In Datura innoxia polyhaploid embroids (2n, 3n, 4n) have been reported to be formed following the fusion of the vegetative and generative cell nuclei, at different stages of DNA replication (Sunderland and Dunwell, 1974). Furthermore, a small number of pollen grains of H niger form chimeral haploid embryoids by divisions of both the vegetative and generative cells (Raghaven, 1976, 1978).

d. Microspore

In one of the more common pathways of androgenesis, mitosis of the microspore nucleus directly leads to the formation of two equal or nearly equal cells of which one or both may participate in callus or embryoid development. This pathway has been observed in Anemonecoronaria, Alopa belladonna and Oryza sativa amongst other species.

Considerable variation in pathways of androgenesis have been found not only in a given species, but even in single anthers. Generally, one of the above pathways predominates, but some pollen grains may follow a second or even a third pattern of development.

Therefore the ploidy of plants derived from anther or microspore cultures is highly variable, and in fact non-haploidy predominates in most species. In N. tabacum most of the plants obtained are indeed haploid, but diploid as well as aneuploid plants are formed.

Non-haploidy is prevalent both in embryogenic and callus-producing speciies and in species with two or three-celled pollen grains irrespective of their hormone requirements. In Citrus aurantium (Hidaka , et al, 1982) only non-haploids have so far been obtained, and in many others non-haploids ournumber hapolids. Examples are Atropa belladonna, Digitalis purpurea, Hyoscyamus niger and Oryza sativa.

The wide range of ploidy levels seen in androgenetic plants has been attributed to nuclear aberrations during division in the pollen itself or the callus derived from it. It is generally assumed, that in the case of callus derived plants that the non-haploids

are generated by endomitosis in the dividing callus cells. However, this can probably only account for diploids and tetraploids. If triploids and pentaploids are generated during callus growth, nuclear fussion is the more likely source. Furthermore, some of the plants derived by anther culture, especially in the callus producing species, could originate from somatic tissues. Also, it is not yet certain that somatic cells are completely eliminated during isolation of pollen from anthers.

1.8 Uses of Haploids

Diploidization of haploids can be achieved by various ways, mainly by doubling the chromosone number by colchicine or by selection of diploids from plants regenerated from callus.

In the last decade, intensive work has been started in many countries to utilise doubled haploids obtained from pollen grains. In Japan, a tobacco variety 'F211' has been produced via anther culture which is more resistant to bacterial wilt. Anthers derived doubled haploid lines of rape are being field tested in Canada. More recently West German workers have produced barley yellow mosaic virus (BaYMV) resistant Hordeum vulgare lines by anther culture (Foroughi-Wehr and Friedt, 1984).

In the above investigations, use has been made of naturally occuring superior traits. Nevertheless, haploid cells offer an opportunity for obtaining new and increased variability by mutations. Various recessive mutations are directly expressed in haploids due to the occurence of only a single copy of genes. Several workers in this field have made use of callus, cell suspensions, and protoplasts from pollen plants for obtaining new mutant lines. Work has been done on the isolation and characterisation of mutants resistant to various metabolic inhibitors, environmental

stresses, herbicides, phytopathotoxins and their analogs (Malaga, 1980). Tyagi, et al (1981a) recently succeeded in obtaining salt-resistant plants of Datura innoxia from a cell line selected from haploid derived callus cultures.

In order to avoid the problem of change of ploidy level in cells, attempts have been made to obtain haploid mutant plantlets, from donor plants which were raised from seeds previously treated with ethyl methane sulphonate(EMS)(Wong, et al, 1983). Mutants have also been obtained by administering the mutagen as early as the pollen mother cell stage, followed by the culture of anthers containing pollen grains.

1.9 Anther Culture of Saintpaulia ionantha

The first report of successful anther culture of Saintpaulia was by Sitbon (1973) cited in a review of anther culture by Dore and Lambert (1973). However, although plantlets were regenerated fromanther callus the origin and ploidy level of both callus and plantlets were not provided (Table 1.1) Hughes, Bell and Caponetti (1975) working at the University of Tennessee were the first to describe the production of haploid plantlets directly from anthers of Saintpaulia ionantha. This initial success was later confirmed by Smith and co-workers at Texas A & M University (Smith, et al, 1981). Two years later a research group in the United Kingdom also reported the production of haploid plants from African violet anthers (Weatherhead, Grout and Short, 1982).

This section of the introduction will be devoted to a review of the above studies in an attempt to identify the conditions required for successful haploid production in Saintpaulia ionantha.

Earlier studies have indicated that callus or plantlet production was only observed in anther containing pollen either in the late uninucleate stage or undergoing the first pollen grain mitosis (Hughes, et al, 1975; Weatherhead, et al, 1982). Although, Hughes, et al (1975) showed that the most responsive anthers were from buds 3-5 mm in diameter, as of yet, no attempts have been made to develop an external staging system which permits rapid identification of suitable anthers. This is probably partly due to the fact that, as bud size varies with the plant variety (Smith, et al, 1981), and previous workers have tended to pool the results of a number of varieties (Hughes, et al, 1975), it has therefore been impossible to identify the appropriate bud stage. Even when a single variety has been under investigation (Smith, et al, 1981; Norris, et al, 1982; Weatherhead, et al, 1982) no indication has been given of the stage of bud development from which the responsive anthers were obtained (Table 1.1).

No research has been undertaken to investigate the conditions under which the donor plants are grown that might influence the response of anther in culture. Donor plants have been grown under a variety of light, temperature and nutritional conditions. These include either growing plants in the greenhouse under natural light (Hughes, et al, 1975; Weatherhead, et al, 1982) or in growth chambers illuminated by Gro-Lux wide spectrum (Sylvania) set at different photoperiods (Smith, et al, 198 ; Bhaskaran, et al, 1983) (Table 1.1). Donor plants have been subjected to growing temperatures ranging from 24-30^{o}C and to different types of fertilizers (Smith, et al, 1981 ; Bhaskaran, et al, 1983).

The research groups in the United States have investigated androgenesis in 26 varieties of African Violet. However, due to the pooling of various varieties and the different conditions they are cultured at, it is impossible to isolate the effect of the Genotypes on the influence of androgenesis. However, two varieties, 'Purple Rhapsodie' and 'Shag' can be compared as they were drawn at the same institute under identical conditions. Data in table 1.1 shows that anthers from 'Purple Rhapsodie' were three times more responsive and five times more productive when compared to those cultured from the variety 'Shag'.

Further evidence onthe role of the donor plant genotype on androgenesis is seen by the work of Weatherhead, et al, (1982). This group working with four commercial lines of the variety 'Blue Rhapsody' described a variation in anther response depending on the cultivar under investigation. Their combined data from 2 experiments shows that cultivar P5 is the most responsive followed by P, B and P2 (Table 1.1).

The only report of the effect of low temperature pretreatment onthe adrogenetic response is given by Weatherhead, et al(1982). They describe that pretreatment of excised flower buds at $7^{o}C$ for 4, 8 and 12 days was inhibitory to callus production.

The medium initially used for successful haploid production was that of Blaydes (1966). Hughes, et al (1975) reported an anther response of 20% with between 1-5 plantlets produced per anther. Further work by Smith, et al (1981), using the smae medium has shown an anther response ranging from 3.6-30% and a similar number of plantlets were produced. (Table 1.1).

However, work by Weatherhead, et al (1982) showed that between 9 and 31% of cultured anthers produced callus and that 50-200 plantlets could be regenerated per anther using solidified MS medium supplemented with NAA:BAP, 1:0.5mgl^{-1}.

Blaydes's (1966) medium contains 5mgl^{-1} of IAA and 0.5mgl^{-1} of kinetin. Weatherhead, et al (1982) discovered that altering the auxin and cytokinin levels to either 0.5:1 or 1:1mgl^{-1} respectively caused rapid prolifieration of callus from the anther wall which overgrew callus produced from the interior and limited callus formation from the region of the severed filaments. However no histological data was given support of their observations.

The employment of liquid media for anther culture was found to be deterioius as anthers sank into the medium and failed to develop (Weatherhead, et al, 1982).

Anthers of Saintpaulia ionantha have either been cultured in 18mm (Hughes, et al 1975), 25 x 150mm (10 anthers per tube) (Smith and co-workers 1981, 1982, 1983) or in 5cm Petri dishes (5 anthers per dish) (Weatherhead, et al, 1982). All cultures to date have been maintained between 23-29^{o}C, under fluorescent tubes (Warm White or Gro-Lux) at a 16 h:8h photoperiod. However, investigations into the effects of temperature, light intensity, quality and photoperiod on the effect of androgenesis have yet to be carried out.

Previous workers have reported that anthers have arisen directly from the anther surface or from a light tan callus (Hughes, et al,(1975). Chromosome counts from root tip cells showed that all plants derived from anthers cultured on Blaydes's(1966) medium

were haploid, n =14 (Hughes, et al, 1975; Smith, et al, 1981; Norris, et al, 1982). Furthermore the same researchers have also reported variation in the haploid chromosome count ranging from 13-15 (Table 1.1). Weatherhead, et al(1982) reported that the majority of plants regenerated from the callus originating from the callus interior were haploid. However, they also reported that 4-24% of plants regenerated from callus were nonhaploid. Neither the American nor the Britisn groups have shown the presence of either multicellular pollen grains or globular, heart and torpedo stage embryoids within anthers that would signify the successful induction of pollen embryogenesis (Bajaj, 1983). Smith, et al, (1981) reported that their haploids appeared to be stable after 2 years. However, after four years they were shown to be diploid (pers. comm., 1983). Bhaskaran, et al (1983) have also shown that haploid plants originating from a previous study (Norris, et al 1982) are now either diploid or tetraploid. The above two studies emphasise the need for frequent screening of anther-derived plants for ploidy since polyploidization by endomitosis seems tobe quite common in African violets.

In 1981 Smith, et al, characterised three haploid plants derived from the same anther. They found that the haploids grew slower and after two years in the greenhouse they were two thirds the size of donor plants. Furthermore, all three plants flowered afgter 18 months in the soil.The haploids were found to be sterile but it was observed that they retained flowers longer than their fertile diploid counterparts. Two years later Norris, et al (1982) phenotypically characterised eleven, 18 month old, haploid African violet plants derived from a single anther. They reported that all the haploid plants were much smaller

than the parent plant (cv. 'Shag'). The smallest was 33% and the average of the rest was 64% smaller. They attributed this size reduction and other phenotypic differences in leaf, margin, indentation and shape as an indication that all eleven plants were derived from individual microspores. Recently, workers from the same laboratory have shown that some anther-derived plants showed higher RuBcase activities, higher than that of the parent plants. (Bhaskaran, et al 1983). This is encouraging as such techniques could be used to screen anther-derived lines from agriculturally important crop plants for increased CO_2 fixation.

Table 1.1 Androgenesis in Saintpaulia ionantha

Date	Authors (Institution)	Saintpaulia ionantha Varieties and Commercial Lines	Growth Conditions of Donor Plants
1973	Sitbon	Saintpualia	
1975	K W Hughes S L Bell J D Caponetti (Tennessee)	Not known but described. 1. Flower purple, double. 2. Flower white, double. 3. Flower pint, double. 4. Flower pale blue, single.	Greenhouse grown. Natural light.
1981	R H Smith M Kamp R S Davies (Texas A & M)	'Purple Rhapsodie' Flower deep purple, single.	Wide Spectrum Gro-Lux (Sylvania) with a 14:10h photoperiod at $27\pm3^{o}C$. Fertilised weekly - N:P:K 20:20:20
1982	R E Norris R H Smith P Turner (Texas A & M)	'Shag' Flower dark purple	Wide Spectrum Gro-Lux (Sylvania) at $30\mu Em^{-2}s^{-1}$.
1982	M A Weatherhead B W W Grout K C Short (Leeds & NELP)	'Blue Rhapsody' 1. Flower purple, signle, 2 anthers(P2) 2. Flower purple, single, 5 anthers(P5) 3. Flower pink,single, 2 anthers (P) 4. Flower blue, single, 2 anthers(B)	Greenhouse grown Natural light
1983	S Bhaskaran R H Smith J J Finer (Texas A & M)	'Shag'	Gro-Lux (Sylvania) 9:15h photoperiod, $28^{o}C$. Fertilised bi-weekly Half-strength Hoaglands.

Table 1.1 continued

Date	Optimum Anther Stage	Medium Composition and Containers	Culture Environment
1973	-	-	-
1975	Late uninucleate - first pollen grain mitosis, ie buds 3-5mm in diameter	Blaydes (1966) Filaments removed. 18mm test tubes	16h photoperiod White fluorescent tubes, 36,000 ergs cm^{-2}, $27\pm1^{o}C$
1981	Not given, but buds 3.5-6mm in diameter used	Blaydes (1966) Filaments removed, 25x150mm test tubes	16h photoperiod Wide spectrum Gro-Lux tubes 24u Em $^{-2}s^{-1}$ $27\pm2^{o}c$
1982	Not given but, buds 4-6mm in diameter used	Blaydes (1966) Filaments removed	16h photoperiod. Wide Spectrum Gro-Lux tubes, 24u$Em^{-2}s^{-1}$ $27\pm2^{o}C$
1982	Late uninucleate	Murashige & Skoog (1962) + NAA (1.0) + BAP (0.5), Filaments removed 5cm Petri dishes	16h photoperiod. Warm White Tubes, (3000 lux). $25\pm2^{o}C$
1983	Not given but buds 3-6mm in diameter used	Blaydes (1966) Filaments removed	16h photoperiod. Wide Spectrum Gro-Lux tubes, 24u $Em^{-2}s^{-1}$ $27\pm2^{o}C$

Table 1.1 continued

Date	Anther Response and Production	Plantlet Ploidy and Origin	Anther Derived Plant Characteristics
1973	Plantlets regenerated from callus	-	-
1975	Plantlets 18% Callus 2% 1-5 per anther	Haploid n=13-15 recorded	Not given
1980	Plantlets 30% (But pooled results of many varieties 3.6-3.8%) 1-5 per anther	Haploid n=13-15 recorded	Slow growing, Leaves $\frac{2}{3}$ size of parent. Flowered after 18 months, retains flowers longer than parent but sterile.
1982	Plantlets 10% (But pooled results of 20 varieties 3.8%) 1.1 per anther	Haploid n=13-15 recorded. Derived from pollen, based on evidence of different Phenotypes	Size reduction 33-64% of parent plant. Variation in leaf margins recorded.
1982	Callus $P_2 11 \pm 1$, P5 22.5 ± 8.5, $P21 \pm 3$, $B12.5 \pm 3.5$ 50-200 plantlets per callus	Haploids 76% Assumed to be from pollen callus. Non-haploids 24%	Not given
1983	4 plants described, but they are from a previous study (Norris, et al, 1982)	3 diploids 1 tetraploid	Variations in:- Guard cell chloroplast Nos, DNA content in flower petal and RuBRCase activities

1.10 Cold Tolerance

The steadily increasing energy prices and the subsequent increases in production costs of glasshouse crops have raised great interest in energy conservation, Various technical procedures, eg the use of thermal curtains, double glazing, etc, have been used to limit energy costs in plant production. However the use of low temperature tolerant genotypes and the use of low temperature adaptions (Went, 1958) have been exploited to reduce the cost of large-scale production of ornamental plants.

Low temperature tolerant genotypes may be obtained in different ways, viz by a) selecting from existing cultivars, b) cross breeding between cultivars and wild species, c) adapting existing and outstanding cultivars through mutation breeding and d) using *in-vitro* techniques (selecting from somaclonal variability and induced mutations).

1.10.1 Adaptation of Plants to Low Temperature by Means of Exploiting Photo-and Thermoperiodic Effects on Plant Growth

Garner and Allard (1920) demonstrated the influence of day length on flowering.They concluded that on the basis of their differing responses to light and dark, flowering plants can be divided into three groups: short-day plants for example, *Xanthium*,chrysanthemums, poinsettias, cocklebur; long-day plants, eg petunias, spinach, radish and lettuce and plants that had no special day length requirements (day-neutral plants), eg cotton, tomato and African violets. However, these divisions, following further study, have proved to be far more complex than originally envisaged by Garner and Allard. There are for example, plants with an absolute requirement for

short-day induction, but others whose flowering is only hastened by short days. Some plants require a combination of day lengths, eg short days followed by long days. Furthermore, there are complex interactions between temperature and day length.

Arthur and Harvill (1937) observed that tomato plants kept in continuous light became abnormal, grew poorly or even died. This phenomenon was investigated in greater detail by Hillman (1956). In their studies young tomato plants were grown in continuous light in rooms with constant temperature. Regardless of the temperature in which they were grown the plants decreased in growth rate, became spindly and the new leaves remained smaller and showed chlorotic spots. Furthermore the rate of development of the apical meristem was slowed down, so that fewer new leaves were formed during the same length of time, whem compared with other plants kept at the same temperature, but in a light/dark alternation. Therefore, it was observed that the deleterious effect of continous light was not so much a question of dark reactions that had to occur in alternation with light reactions, as it was a question of rhythms, for two reasons. Firstly, Hillman(1956) found that the dark interuption was effective only if presented on a 24-hour cycle. Secondly he found that plants in continuous light can develop normally when they are subjected to a 24-hour cycle in temperature. Thus an 8-hour light period at $10^{o}C$ is equivalent to an 8-hour period of darkness if, during the other 16 hours, the plants are kept at 20 or $23^{o}C$.

Working with peas, Went (1959) found this equivalence of a succession of light and darkness with a temperature fluctuation. He concluded that for optimal development of a plant, there must be a 24-hour cycle in the external environment, even though the plant itself does not

show any rhythmicity under constant external conditions Therefore the tomato or pea plant has an internal periodicity (circadian rhythm) which requires an external 24-hour signal for either synchronization or expression of this rhythmicity.

Tomato plants have been shown to grow as well in a 24-hour light/dark cycle under constant temperature whether the light period is provided during day or night hours. Thus, this makes it unlikely that some external cycle with a 24 hour period, such as cosmic radiation, must be synchronized with light and dark periods, and means the plant rhythm must be autonomous.

In a later series of experiments Went (1962) found that the autonomous circadian rhythm is so strongly ingrained in plants that they are unable to adjust themselves to an external daily rhythm other than their internal rhythm. Went demonstrated this for a number of different plants in a phytotron, in which both temperature and cycle length could be regulated independently of each other, and could be maintained for many months in succession. In half a dozen plants it was demonstrated that their internal rhythm had a temperature coefficient of about 1.2 and that the length of circadian rhythms can be seen as a function of temperature. Therefore, a Californian spring annual, Baeria chrysostoma, dies in a light:dark, 12:12 hour cycle at 26°C, but can support this elevated temperature on an 18-hour cycle. (9 hours light followed by 9 hours dark). Similarly, Saintpaulia ionantha, a tropical plant, which at 26°C has a cycle of 24 hour, dies within several months at 10°C, unless kept at that temperature on a 32-hour cycle length. At this cycle length African violets will undergo normal vegetative and reproductive development.

1.10.2 Breeding for Cold Tolerance

Modern cultivars of the major crop and ornamental species are well adapted to controlled cultural practices, but are not highly tolerant of temperature extremes. Temperature stress is the major factor affecting plant growth and development and therefore, crop yields. Since plant tolerance to temperature stress is heritable, selection and breeding can be used to improve the trait. However, major limitations in selecting for cold tolerant varieties are the lack of effective selection and breeding technology and the apparent narrow ranges of genetic diversity in the gene pools of several crop species. Regardless of this some important successes have been recorded in wheat, barley and oats. Similarly, improved cold tolerance has been introduced to the forage crop alfalfa.

Chilling sensitive species are in general of tropical origin and they can be found in taxa as diverse as Solanaceae (dicot.) and Musacaea (monocot). Some effort has been made towards introducing and increasing cold tolerance of chilling sensitive cultivated plants. In general the accomplishments in these efforts have not been spectacular. Plant breeders have focussed on seed germination at low temperatures to enable the early planting of crops. Selection for seed germination at low temperatures have been made in cotton, soybean, beans, maize and tomato. In almost all cases cold tolerant lines were selected from cultivars of the above crops.

Comparisons and selection of cultivars for their tolerance to low temperatures during different stages of development (other than germination) have been obtained with a varying degree of success in: tomatoes, during a whole life cycle, and fruit set; maize, growth in

general; cotton, growth development and boll maturation and chrysanthemum, rooted cuttings grown to flowering.

Another option open to breeders is to introduce cold tolerance from wild species or primitve forms of a species. Such work has been attempted in sorghum (Van Arkel , 1977) and tomato(Vallejos, 1979). In both cases highland races have been used to introduce cold tolerance into the cultivated plant.

1.10.2.1 Breeding for Cold Tolerance in Saintpaulia

The commercial Saintpaulia is primarly the result of extensive breeding and selection only within a single species S.ionantha Wendl. (Arisumi, 1964). The genetic reservoir of the 18 other varieties still remains largely untapped despite breeding experiments (Clayberg, 1961) revealing practically no barriers to hybridization among most species. Only S.grotei and S shumensis have been used, although to a minor extent, for development of trailing and miniature Saintpaulias respectively. (Bilkey, Phd Thesis, 1981).

In 1981 Bilkey successfully crossed S ionantha var. Georgia (a species that grows and flowers optimally at 20 - 25^{o}C) with S. shumensis (a species that is native to much higher attitudes where the temperature can fall below 7^{o}C in the winter). Tests were run on the resulting hybrid over a period of 102 days at various temperatures (20^{o}C, 13^{o}C, 11^{o}C) and the results confirmed that the performance of the hybrid was significantly better under cool conditions than its commercial Saintpaulia parent (Georgia). Subsequent tests at 28^{o}C showed that the F1 hybrid did not grow as well as the commercial variety indicating that optimum growth was achieved at lower temperatures. Subsequent outcrossing to a wide range of commercial varieties has now resulted

in plants of an acceptable size and marketable quality. S. shumensis itself, is a miniature variety with very pale flowers and two generations of further seedlings were required before progeny of sufficient marketable quality were obtained.The cold preferent varieties were put on sale to the public from the winter of 1983 under the Generic title 'Endurance'.

1.10.3 Mutation Breeding for Low-temperature Tolerant Cultivars

An increased degree of winter hardiness has been observed in some mutants of Hordeum vulgare, Triticum aestivum, Secale cereale and Oryza sativa (Enchev, 1976; Khvostova, 1967; Kivi, et al., 1974; Kawai, 1967) Tifway-2 Bermuda grass, released turf grass variety obtained by gamma irradiation, was found to be not only more frost resistant but showed a better increase in spring growth. The tremendous expenditure, necessary for reaching a distinct aim of breeding becomes abundantly apparent when it is realised that 500,000 green stems with dormant buds of coastcross-1 Bermuda grass were treated with gamma rays, giving rise to one single mutant with improved winter-hardiness. However, in Finland, mutants of Brassica campestris oleifera have been selected, surpassing the mother variety with regard to winter-hardiness. Furthermore, an improved frost resistance has been observed in an X-ray induced sour cherry mutant. Recently Broertjes et al (1983) was able to select low-temperature tolerant cultivars of Chrysanthemum morifolium following irradiation of cuttings with X-rays.

No studies have been undertaken to induce cold-tolerant mutants from vegetatively propagated African violets. However, Saintpaulia has been used as a model plant for assessing mutagenic agents by many research

groups for the reasons that will be described below.

A very effective method, important in practical aspects, with regard to the performance of mutation breeding in vegetatively propagated species is the so-called adventitious bud technique (Broertjes, 1969,a, c; 1972, b). Many plant species can be stimulated to form adventitious buds on isolated leaves. These buds originate very often from a single meristematic cell. If mutational events have been induced in this cell, the solid mutants can be produced by the known totipotency of this species.

Sparrow et al (1960) found that irradiation of Saintpaulia leaves either resulted in the majority of cases in the formation of solid mutants or of completely normal plantlets developing at the base of the petiole. This confirmed the early work of Naylor and Johnson (1937) who concluded from a histological study that plantlets were formed from only one epidermal cell. This system has been used by Broertjes (1968) to study the effect of dose rate and dose fractionation of various ionising radiations upon survival of leaves, production of plantlets and number of plantlets. He discovered that after irradiation almost all plantlets turned out to be non chimeric viz they are their completely normal or complete solid mutants. Broertjes concluded that since the diplontic selection is reduced to a minimum the mutation frequency is high for X-rays (30% optimum acute dose rate of 5 Krad). Khokhar et al (1982), also using X-rays produced novel African violets with speckled, bicolour flowers.

Chemical mutagens have also been used successfully in African violet mutation breeding. In 1973 Warfield used the mutagen Ethyl Methane sulphonate (EMS) on callusing leaf petiole bases of African violet. He found that

a dose of 0.5 EMS for one hour produced 13.4% mutants. In the same year Broertjes reported a high percentage of solid non-cytochimeral polyploids formed after leaves were treated with colchicine. The following year East German workers reported that leaves treated with N-nitrose-N-methyl urea (NMU) regenerated 30 per cent chlorophyll variants (plastid mutations) while another 11% showed variations as to the colour and shape of the flower and leaves and growth type. (Hentrich and Beger, 1974; Polheim and Beger, 1974).

Work by Warfield et al (1975) showed that X-ray treatment of petioles can increase peroxidase activity. Following on from this work Kelly and Lineberger(1981) showed that such activity can be used as an effective genetic marker to detect variants that would otherwise be missed using morphological criteria.

In 1980 a technique was developed which permits a study of the effect of radiation on Saintpaulia leaves at the cellular level in the epidermal cells at the base of the petiole, which eventually grow into plantlets (Engels et al, 1980).

Broertjes (1972) found that a small dose of radiation (pre dose) given 8-24 hours before the challenging radiation dose made the Saintpaulia much more resistant to the challenging dose than unpretreated leaves. Recent work by Leenhouts and co-workers (1981, 1982) at the cellular level has indicated that the pre-dose renders the stationary epidermal cells more able to repair potentially lethal damage. The effect of the pre-dose stimulated repair on the induction of mutations in the plantlets derived as adventitious shoots from the irradiated leaves has been studied. The above workers concluded that the stimulated repair tends to be error free, although not completely.

1.10.4 Selection for Cold Tolerance by In-vitro Means

1.10.4.1 Somaclonal Variation

Plants regenerated from undifferentiated tissue cultures have become a new and useful source of genetic variation. Such variation generated by the use of a tissue culture cycle without the application of known physical or chemical mutagens has been termed as somoclonal variation by Larkin and Scowcroft (1981). They defined a tissue culture cycle as a process that involves the establishment of a "differentiated cell or tissue culture under defined conditions, proliferation for a number of cell generations,, and the subsequent regeneration of plants".

Plants regenerated from shoot apex cultures may occasionally include variants; however, most reports describing plants form such cultures contend that genetic stability is preserved. The shoot apex provides a structure in which cell division and DNA replication are strictly controlled, and those cells with impaired reproductive ability are eliminated because of competition.

Cultured cells, however, are not usually genetically stable. Polypolidy, aneuploidy, and chormosome structre changes in cells in culture under various conditions have been extensively described (Bayliss, 1980; Sunderland, 1977). Variation in genetic composition is also known in whole plant tissues (Murashige and Nakano, 1966; Neilson-Jones, 1969). Therefore it is not surprising that many of the plants regenerated from undifferentiated cultures (callus, cell suspension, and protoplats cultures) are altered. For the purpose of germplasm preservation

and plant micropropogation the inherent stability of shoot apex cultures are requied. But the diverse variations characteristic of plants obtained from undifferentiated cell cultures might be of great use to plant breeders.

Plant somaclonal variation generated by the use of a tissue culture cycle may be encouraged by six different techniques, namely: (1) long-term culture cycle (eg Chrysanthemum morifolium; Sutter and Langhans, 1981), (2) a protoplast culture cycle (eg Solanum tuberosum sbsp tuberosum cv.'russet Burbank'; Bidney and Shephard, 1981), (3) a callus culture cycle (eg Oryza sativa; Oono, 1981), (4) the use of explants from specified tissues (eg Ananas cosmosus, Wakasa, 1979), (5) the generation of random variation concomitant with the selection of a specific nutrtient medium or hormone formulation (eg Hordeum vulgare, Deambrogio and Dale, 1980), (6) the use of certain genotypes that tend to produce increased amounts of variation (Pelargonium sp, Skirvin and Janick, 1976a).

Explants used in this process may come from virutally any tissue including leaves, internodes, ovaries, roots and inflorescences. But the above classifications are broad in scope and are not mutually exclusive. As an example, the use of a long term tissue culture or a specific explant source must be done in conjunction with either protoplast or callus culture.

1.10.4.2. Induced Mutation in Tissue Cultures

The frequency of genetic variation can be increased *in vitro* by treatment of tissues or cell cultures with known physical and chemical mutagens. These are capable of increasing the frequency of changes in the genetic material when the cultures are placed under conditions which allow for the rapid screening of the mutants. However, mutations are considered true only when, from mutated tissue or cell lives, one can regenerate plants capable of producing similar mutated individuals.

The variation of approach in *in vitro* mutation breeding is well demonstrated by the different choice of systems available for mutagenesis , namely:-

a) Protoplasts: before or at first division
b) Cell suspension: individual cells or clumps (in log phase of growth)
c) Callus cultures: in log phase of growth
d) Adventitious buds from original explant or from callus material
e) Anther at microspore uninucleate stage
f) Shoot tip or meristem culture
g) Auxillary bud: at initiation of meristem development
h) Explants of various plant organs or tissue before or during primary *in vitro* culture

Furthermore the aim of successful mutagenesis has been to develop methods which give the highest rate of gene mutations with the lowest chromosome and physiological damage. Both physical and chemical mutagens have advantages and disadvantages when used in conjunction with tissue cultures.

Physical Mutagens

Several kinds of radiation are potentially useful mutagens in tissue culture systems, such as ionizing radiations (eg X-and gamma rays) (King, 1949; Bajaj, 1970) and ultra violet (UV) light (Eriksson, 1965). As a result of the interaction between radiation and matter, atoms and molecules are excited or ionized. In the case of UV radiation, the bulk of the energy is transferred, to the living systems, in the form of excitaton of nucleic acids. However, in the case of ionization radiation, several kinds of events occur such as disruptions to water molecules, DNA, enzymes, growth substances, etc, leading to disturbances in the homeostasis in the cell.

The degree to which cell and tissue cultures are affected depends on; the type and amount of radiation the environmental conditions prior to, during and after irradiation (temperature, light, oxygen); and the kind or stage of the material which is being irradiated (plant species, ontogenetic stage, ploidy level, phase of the cell cycle, etc).

One of the major advantages of using physical as opposed to chemical mutagens is the high degree of penetration in multicellular tissue systems (although not valid for UV). However, one of the disadvantages is the possibility of sterility in plants regenerated from irradiated cultures.

Chemical Mutagens

Compared with physical agents, chemical mutagens are perhaps more capable of leading to specific and predictable changes involved in a mutation, on account of the direct chemical action of such compounds as DNA molecules. Some effects of chemical mutagens

include; the alteration in the sequence of nucleotides (substitutions direct changes in the nucleotide structure, such as the removal of the amino group from adenine and cytosine, the addition of new chemical groups to the bases, leading to new possibilities in the pairing or replication, or even inactivation of DNA (Broertjes and Van Harten, 1978). The most commonly used chemical mutagen, ethyl methylmethanesulphonate (EMS), belong to the group of alkylating agents, that act by adding methyl or ethyl groups to guanine which can thus behave as a base analogue of adenine, with production of pairing errors. Other chemical mutagens used include; N-methyl-N-nitro-N-nitrosgua - idine(NG), N-ethyl-N-nitrosourea (ENUE). Methyl-nitroso-urea (MNUA), N-nitroso-N-methylurea (NMH), Di-ethylsulphate (dES), Ethyleneimine (EI) and Sodium azide (SA). (Skirvin, 1978; Maliga, 1980). Chemical mutagens have the following advantages when compared to physical mutagens: 1. point mutations predominate; 2. less chromosome damage; 3. high mutation rates are known in certain systems. However, one disadvantage of chemical mutagens is the penetration difficulties found when using multicellular systems.

1.10.4.3. In Vitro Selection for Cold Tolerance

The selection of tolerant cell lines by in vitro methods has been reported by several workers. This work has included selection for resistence to herbi-cides (metribuzin, Glycine max, Oswald, et al (1978) Hordeum vulgare, Collin, et al, 1984), pathotoxins and or diseaseas (Phytophthora infestans-Solanum tuberosum; Behnke, 1979) and salt tolerant lines (N tobacum; Nabors, et al, 1980)

Cell lines with increased cold tolerance have been isolated in N. sylvestris (Dix and Street, 1976; Dix, 1977) and Daucus carota (Templeton-Somers, et al, 1981). Although plants were regenerated from

three lines of N. sylvestris with a low level of cold tolerance (Dix, 1977), the phenotype was not transmitted sexually. Templeton-Somers et al (1981) reported that although cold-tolerant callus, derived from mutagenized cells (1% EMS, 1h), of D. carota was isolated, this trait was not expressed in embryos. This instance emphasizes the problem that not all traits selected in unorganised callus cultures will be expressed in the organised differentiated plant. In 1982 Chen et al, reported cold-tolerant cell lines of Saccharum cv F156. However although the lines retained cold tolerance for a number of weeks no plant regeneration was recorded.

Recently, Preil, et al, (1983) regnerated low temperature-tolerant lines from x-irradiated Poinsettias and Chrysanthemums. In experiments to confirm the mutatively changed nature of the few selected plants following clonal propagation, three poinsettia clones and two selected chrysanthemum clones proved to be real physiological low-temperature tolerant mutants.

1.10.4.4 In Vitro Mutagenesis and Low Temperature Tolerance in African Violets

Jungnickel (1977) first described the induction of African violet mutants using tissue culture techniques. Mutant lines were regenerated from shoots or calli derived from axenically cultured leaf cuttings treated with N-methyl-N-nitroso-urea. Most of the mutants differed from the wild form in growth type. (eg reduced apical dominance) or in size shape or colour of the leaves. Three years later Grunewaldt, using tissue culture techniques, reported two spontaneous flower colour mutants of African violet. These plants differed in flower colour from the mother plants and from each other . Furthermore, chlorophyll mutants

and two plants with an altered flower colour resulted from mutagenic treatment of leaf pieces with N-nitroso-methylurea. Espino and Vasquez (1981) studied the effects of chemical mutagens on cultured fragments of leaves of African violet. They used colchicine and caffeine as a supplement to the MS medium. The discovered that the rate of regenerated polyploid plantlets was lower in caffeine as compared with colchicine. In both cases a high percentage of cytochimeric plants was observed. The chimeras were recognisable due to the variation in chromosome number and interphasic nuclei of different sizes. Further studies by Grunewaldt (1983), using cultured leaf explants derived from leaf, blades treated with N-methyl-nitroso-urea showed that 40% of the regenerates showed pigment alterations in the early leaf stages. The alterations ranged from green to light green, yellow, dish or blueish. Grunewaldt also reported unchanged homogenous green leaves but a changed flower colour. These plants possessed a pela flower and when propagated vegetatively they main this colouration.

Schlegel (1982) studied the influence of low temperature on regeneration of African violets. In three experiments leaf cuttings and in vitro cultures of 30 Saintpaulia clones were subjected to different temperature treatments before or during regeneration. He discovered that temperature level, genotype, and preculture of donor plants (in vitro/in vivo) had significant effects on survial and regeneration of cultures. Leaf cuttings died after treatments for 96h at temperatures below 6°C. At 6°C only part of the cuttings survived and regenerated and differences between clones were observed. Explants cultured in vitro for 16 weeks showed no regeneration at temperatures below 15°C, although a few clones survived

at 12°C. At 15°C regneration was strongly delayed, the growing shoots were partially chlorotic. Only 30% of clones responded at this temperature. However, explants originating from in-vitro cultured donor plants showed better survival at low temperature than explants from greenhouse-grown donor plants. During a period of 7 weeks at 12°C the latter became completely necrotic, while explants from in vitro-cultured donor plants were still able to regenerate shoots after transfer to standard growing conditions. Such results are important indicators for work on the selection of low-temperature tolerant mutants.

Preliminary work on the induction and selection of low-temperature-tolerant mutants in tissue cultures of Saintpaulia by Geier (1983) indicated the following:
(1) Using segments of the leaf lamina as a unit for selection and mutation studies is acceptable as histological observations confirmed that in Saintpaula adventitious shoots generally arise directly from single epidermal cells. Furthermore, the epidermis of a leaf segment of 8mm square is composed of 10,000-20,000 cells. Most of the cells can be induced to divide but due to competition effects relatively few finally develop into shoots. It can be expected, however, that induction of mutants having a selective advantage over wild type cells will determine the patterns of shoot formation in the way that preferably mutant cells will be able to regenerate shoots. Therefore the actual size of population being available to selection probably is close to the total mumber of epidermal cells of the explant.
(2) Comparative studies were made on the effect of ethylmethane sulphonate (EMS) and N-nitroso-N-methylurea (NMH). Mutagenic effectiveness and efficiency was calculated from the rate of chlorophyll-deficient

adventitious shoot and from the degree of inhibition of shoot formation. NMH was found to be generally 250-1000 more effective and 3-10 times more efficient as compared to EMS.

(3) In preliminary experiments on the selection of types with reduced temperature requirements leaf segments of Saintpaulia were cultured at 5^{o}, 10^{o} and $13^{o}C$. After 4 weeks, $5^{o}C$ proved to be lethal. Ten and $13^{o}C$ did not permit growth, however, after transfer to $26^{o}C$ delayed regeneration occured and finally reached about the same level as in cultures not subjected to low temperature stress. In a subsequent experiment explants of five different cultivars, either untreated, or after mutagenic treatment with $500mgl^{-1}$ NMH for 1h, were incubated at $16^{o}C$ for a month. Geier (1983) discovered that two of the cultivars did not show any regeneration, the three remaining produced 0.35,1.0 and 3.05 shoots per NMH treated culture and 0.9, 0 and 1.6 shoots per control, culture, respectively. Therefore in the last two cultivars, mutagenic treatment, though inhibitory to shoot formation at $26^{o}C$, resulted in a higher number of shoots as compared to the controls.

However, as of yet it is not known whether this higher tolerance to low temperatures is due to heritable changes and/or physiological adaption.

In conclusion Geier (1983) proposes a scheme for the in vitro selection of cold-treatment lines of Saintpaulia. By exposing explants to selection systems with alternate period of stress and optimum temperatures could restrict physiological adaption and thereby result in more clear separation of mutants and epigenetic variants.

1.11 Aims of Investigation

The aims of this study is to investigate the *in vitro* selection of cultured African violet plantlets for their ability to tolerate low temperatures. The ultimate objective being to produce, from commercialy important cultivars, a variety which is tolerant to low temperatures and thereby reduce the production costs of this plant.

A range of cultured material is used for this study and includes anther, pollen and leaf disc explants. Mutagenesis has been employed in an attempt to increase the selection *in vitro* of cold-tolerant plantlets.

Each chapter clearly sets out its aims and discusses the results obtained.

CHAPTER TWO

MATERIALS AND METHODS

2.0 MATERIALS AND METHODS

2.1 Plant Material

The Saintpaulia variety used in this study was the commercial line Blue Rhapsody supplied by Double H Houseplants, New Milton, Hampshire. The average rossette diameter of the parent plant was 215±8.8mm. The leaves of this particular variety have a mid-green upper surface with light purple underside on young leaves and a light green underside on older leaves. The leaves are ovate and slightly crenate with a mean length of 56.6mm and width of 51.5mm. The mature plant has a single, deep purple flower each containing five anthers.

The cold-tolerant "Endurance" variety Wilson used in this study was supplied by Thomas Rochford and Son, Broxbourne, Herts. This variety at maturity is medium sized with a mean rossette diameter of 220±15mm. The leaves are olive green with an average length of 70.6mm and width of 57.4mm. The mature flower is single and has light violet frilled petals.

2.1.1 Vegetative Propagation

Leaf cuttings were taken from stock plants. Mature leaves, approximately 6x5cm, with petioles about 2cm in length were removed. An incision of about 0.5cm was made in the petiole base prior to planting in seed trays containing Vermiculite. After 5-6 weeks, shoots appeared at the Vermiculite surface. Cuttings were maintained in high humidity and after 8-10 weeks adventitious plantlets were removed and transferred to 6cm diameter pots containing peat potting compost (J. Arthur Bowers Ltd).

All stock plants and cuttings were grown throughout the year in the Polytechnic glasshouse with a minimum winter temperature of 18°C. Day length was maintained in winter at 16h

with the aid of supplementary illumination from Thorn Grolux (65/85w) fluorescent tubes at a light intensity of 100 umol $m^{-2}s^{-1}$. Plant material was placed on capillary matting, watered daily from below and given a nutrient feed of Hoagland's solution at weekly intervals.

2.2 Preparation of Tissue Cultue Media

For the majority of experiments a packaged formulation of Murashige and Skoog (MS), (1962) basal medium was used (supplied by Flow Laboratories, Irvine, Scotland). However in experiments where the media required was not available in packaged form it was prepared from analytical grade reagents.

MS medium was prepared by dissolving the contents of a sachet of MS powdered medium (suitable for 1 litre) together with 30g of sucrose in 500 ml double distilled H_2O. The contents were then made up to a one litre volume before the pH was adjusted to 5.8 using 1N NaOH or 1N HCl. The medium was dispersed into two 1000ml, Pyrex Erlenmeyer flasks each containing 4g of Difco Bacto agar. The flasks were capped with silver foil before autoclaving at 121°C (15psi, 103.5K Pa) for 15 min. After cooling, the media was either poured, under sterile conditions, into appropriate containers or labelled and stored at 2-8°C in the dark for future use. Details of the requirements of all the nutrient media used throughout this study are given in Appendix 1. Supplements were added to the medium prior to adjusting the pH and making up to the final volume.

2.3 Aseptic Manipulation and Initiation of Cultures

All Aseptic manipulations and initiation of cultures were carried out in a sterile environment provided by a laminar flow work bench. (Inter Med, Microflow Horizontal Laminar Flow Work Station, Model N° 21957). Prior to any manipulations all work surfaces were wiped clean with 70% ethanol. Throughout the manipulation sequence forceps,

scalpels and other small instruments were kept in 95% ethanol and flamed thoroughly before use.

Manipulations of tissue cultures were usually carried out in sterile Petri dishes. The dish lid was always replaced after each step of the procedure. The prepared explant was then transferred to the appropriate media for culture.

2.4 Anther Culture

2.4.1 Temperature-stress Bud Pretreatments

In some experiments, the effects of temperature-stress bud pretreatments were tested. Saintpaulia buds were harvested, grouped into age categories and placed in polythene bags. The bags were then wrapped individually in thin aluminium foil to minimize water loss and stored in cooled incubators (Gallenhamp Ltd) at $5\pm1^{o}C$, $8\pm1^{o}C$, $15\pm1^{o}C$ and $20\pm1^{o}C$ for varying lengths of time (2-28d).

2.4.2 Pollen Ontogeny

In each experiment a representative sample of anthers was taken from each batch of buds and examined to assess pollen development. On each sampling occasion the constituent pollen was stained in acetocarmine (4% w/v) and a 100 grains were examined under high power using a Leitz Dialux (20EB) light microscope. Acetocarmine stain was prepared by refluxing 4g carmine in 100ml of 45 per cent v/v acetic acid for 24h. All preparations made with acetocarmine were left for at least 30 min before they were scored. Anthers were squashed directly in the stain without prior fixation. Meiotic stages and tetrads were found in anthers taken from buds with breadth of 2.3 ± 0.4mm and 3.4 ± 0.2mm respectively. Anthers from buds of 5.5 ± 0.4mm contained uninucleate grains, while those taken from buds with breadth of 7.4 ± 0.4mm contained grains

undergoing the first pollen mitosis. At this stage the petals were starting to open to reveal the enclosed anthers. Bud-opening was accompanied by deposition of starch in the pollen grain prior to anthesis as seen in buds with breadth of 9.4mm or more.

2.4.3 Pollen Dimorphism

A sample of mature undehisced anthers were selected and squashed in a drop of 4% (w/v acetocarmine.) Any debris was removed before a coverslip was placed gently over the stained pollen. The slide was then heated gently over a flame of an alcohol burner for 30 seconds. The slide was observed under low magnification of a light microscope and scored for the presence of grains of differing sizes and staining characteristics.

2.4.4 Pollen Viability Counts

Pollen viability counts were taken from anthers of all bud classes before culturing and at daily intervals for the first 7 days. Thereafter sampling was at weekly intervals. One anther was selected, from each of five buds, and squashed in fluorescein diacetate, at a concentration of 0.1mg/ml (w/v), for 15 minutes. One hundred grains were scored for the presence of live (yellow/green) or dead (dark green) pollen.

2.4.5 Anther Culture Technique

Buds at different stages of development were excised and surface sterilised for 5-10 min in 5% (v/v) solution of sodium hypochlorite (0.72% w/v available chlorine) to which two drops per 100ml of the surfactant Tween-80 (BDH Chemicals Ltd, Poole, England) had been added. They were then rinsed three times with sterile distilled water before the anthers were isolated aseptically. The anther filaments were removed, with the use of two fine-pointed forceps, and the unbroken anthers were

transferred to solidified culture medium contained either in 5cm plastic Petri dishes (Sterilin Ltd, Teddington, Middlesex) (10ml, 5 anthers per dish) or 30ml universal containers (10ml, 5 anthers per universal). The dishes and universals were sealed and incubated in the growth room.

2.5 Pollen Culture

2.5.1 Hanging-drop Culture Technique

Fifteen to twenty surface sterilized buds were placed in a small beaker containing 20ml of MS basal medium. The pollen grains were then squeezed out of the buds by pressing them against the side of the beaker with a glass rod. Bud and anther tissue debri was removed by filtering through a nylon sieve with a pore diameter slightly wider than the diameter of the pollen (25 μm). The pollen suspension was centrifuged at low speed (500-800 rpm for 5 min) to spin down the pollen. Following the removal of the supernatant the pollen pellet was resuspended in fresh medium and washed twice to remove any traces of possibly inhibitory substances.

The pollen prepared by the above procedure was mixed with varying concentrations of MS medium at a density of 10^{-3} - 10^{-4} grains per ml (7-10 drops pipetted into 5cm wide Sterilin Pertri dishes containing 5ml of medium). The dishes were lightly swirled to disperse the pollen grains into suspension. Fifteen to twenty drops were pipetted from the pollen suspension and plated on the underside of the Petri dish lid. For control treatments drops were plated direct onto the Petri dish lid without prior mixing with the MS medium. The Petri dish lids were gently replaced and each dish sealed with Parafilm to avoid dehydration. Cultured pollen was maintained in the growth room at 25°C either in the light with a 16h photoperiod or in the dark.

2.5.2 Anther Float Culture Technique

Pollen was cultured by using the technique of Sunderland and Roberts(1977). This method exploits the fact that, in many species (Saintpaulia included), anthers dehisce soon after inoculation. Therefore if they are floated on liquid medium pollen is shed into the medium.

Anthers of increasing age were excised from surface sterilized cold-treated and untreated buds of Saintpaulia. To obtain an inoculation density of 10^3-10^4 pollen grains per ml, 12 anthers were floated on the surface of 5ml of A medium or A medium supplemented with a glutamine, serine and myo-inositol (AGSI medium) as recommended by Nitsch(1974). Solidified A and AGSI medium were used for control treatments. All dishes were sealed with Parafilm and incubated at 28°C in the darkness for the first 14 days. After 14 days, they were transferred to light (Pluslux, 16h-day, 40-45 umol $m^{-2}s^{-1}$) at 25±1°C as recommended by Sunderland and Roberts (1977).

2.5.3 Pollen Nurse Culture Technique

Twenty-five anthers, obtained from surface sterilized buds, were placed into a 5cm Petri dish containing 5ml of modified MS (1962) liquid medium. The anther walls were teased with dissecting needles to release the pollen grains. An appropriate aliquot of this pollen grain suspension was transferred into a sterile volumetric flask to form a final pollen suspension of 10^3-10^4 grains per ml of liquid medium for use as nurse culture inoculum.

Nurse cultures were prepared as follows: Intact anthers were placed horizontally on top of solidified basal medium contained within each 5cm Petri dish. A sterile filter paper disc was placed over the anthers. One ml of final pollen suspension was plated on the filter paper disc. Controls were prepared in the same manner, but the pollen grains were plated

on a filter paper disc placed directly on agar. All cultures were sealed with Parafilm and incubated to the growth room (Pluslux, 16h-day, 40-45 $umol^{-2}s^{-1}$) at $25\pm1^{o}C$.

2.6 Leaf-disc Culture

Freshly excised leaves were rinsed in distilled water to remove surface soil particles. Leaves were bathed in 70% ethanol for 30s, then immersed in 10% (v/v) solution of sodium hypochlorite (1.4 % w/v available chlorine). The leaf tissues were rinsed four times with sterile distilled water, before 1cm leaf discs were 'punched out' and placed with the upper surface in contact with solidified MS medium supplemented with 1-naphthylacetic acid (NAA; 0.5 mgl^{-1}) and 6-benzylaminopurine (BAP; 0.5 mgl^{-1}). Cultures were sealed with Parafilm and incubated in the light (16h-day) at $25\pm1^{o}C$. After 4-5 weeks leaf-disc explants were transferred to basal MS medium, to eliminate the effects of growth regulators, prior to further experimental manipulations.

2.7 Plantlet Culture

Shoot primordia were derived from either anther or leaf-disc explants cultured for 55-70d on MS medium supplemented with NAA and BAP various concentrations. Plantlets were formed by transferring ca. 1cm high shoots, at the 4-8 leaf stage, to hormone-free MS medium contained in 60ml Sterilin containers (25ml medium per container). On this medium the shoots quickly developed a normal root system and in 4 weeks had grown to a height of 3cm. All cultures were incubated at $25\pm1^{o}C$ in Pluslux light (16-h day, 40-45 $ummol^{-2}s^{-1}$)

2.8 Maintainance of Growing Tissues

2.8.1 Anther Cultures

Anthers maintained on agar at $25^{o}C$ were sub-cultured onto

fresh MS medium (25ml medium in 10cm Petri dishes), without hormone supplements, every 4-6 weeks. During the early stages of anther-callus production it was convenient to transfer the whole anther tissue. Whereas established tissue (8-10 weeks) required frequent sub-division and transfer of separate shoots (ca 1cm) to either shoot multiplication (MS, NAA; $1mgl^{-1}$ and BAP; $1mgl^{-1}$) or rooting medium (hormone-free MS medium) prior to transplanting to regenerate whole plants.

2.8.2 Leaf-disc Cultures

Adventitious buds appeared after about 5 weeks in culture. At this stage the explant was sub-divided into small pieces (ca $1cm^2$) which were transferred to shoot multiplication media (M, NAA; $0.5mgl^{-1}$, BAP;$0.5mgl^{-1}$) After a further 5 weeks culture shoots were either subcultured onto fresh multiplication medium or transferred to rooting medium (hormone-free MS medium) in readiness for transplanting.

2.8.3 Plant Cultures

Within 8 weeks of culture on basal MS medium each shoot had formed up to 12 lateral shoots derived from axillary buds. Each shoot could be detached and in turn induced to form more shoots by subculture to fresh medium.

2.9 Transplanting of Plantlets to Greenhouse Conditions

After about 5 weeks of culture on hormone-free rooting medium, young African violet plantlets, with healthy root systems, were planted in Jiffy-7 peat pellets (Jiffy Products Ltd, Grorud, Norway) and maintained in mist chamber for approximately six weeks. The plantlets were then transferred to 9cm diameter pots, containing peat potting compost (J Arther Bowers Ltd) and removed to the growth room. After a further period of six weeks plants were repotted in peat potting compost

contained in 12cm pots and transferred to greenhouse conditions. Mature plants were obtained 20 weeks after transfer.

2.10 Growth Conditions

2.10.1 In-vitro

The cultures were incubated either in the growth room at $25\pm1^{o}C$ or in Gallenkamp cooled incubators adjusted to temperatures of $5\pm1^{o}C$, $8\pm1^{o}C$, $15\pm1^{o}C$, $20\pm1^{o}C$ and $28\pm1^{o}C$. Light was provided by 36w white fluorescent tubes (Pluslux 3500, Thorn Ltd, London, UK), at an intensity of 40-45 umol $m^{-2}s^{-1}$ (20cm from cultures), and day length of 16h. Cultures maintained to the growth room were placed on clear Perspex shelving to allow light to penetrate the plant tissues from all directions. However cultures grown in the refrigerated incubators received light from the upper surface only. Dark grown cultures were wrapped in Bacofoil before placing into the growth room or incubators.

2.10.2 In-vivo

Plants, other than those grown in the glasshouse, were maintained under growth frames and illuminated by 36w white fluorescent tubes at an intensity of 60 $\mu molm^{-2}s^{-1}$ (16-h day). Plants were incubated at $10\pm1^{o}C$, $15\pm1.5^{o}C$ and $25\pm1^{o}C$; watered daily and given a weekly nutrient feed of Hoagland's solution.

Extended Photoperiods

In certain culture and plant experiments the duration of the light/dark cycle was extended beyond 24h. Therefore an optimal cycle length was calculated which was dependent on the temperature plants and cultures were grown at. (Fig. 2.1 Data of F W Went, 1960). To accommodate this, growth frames were fitted with fully electronic, digital and highly accurate

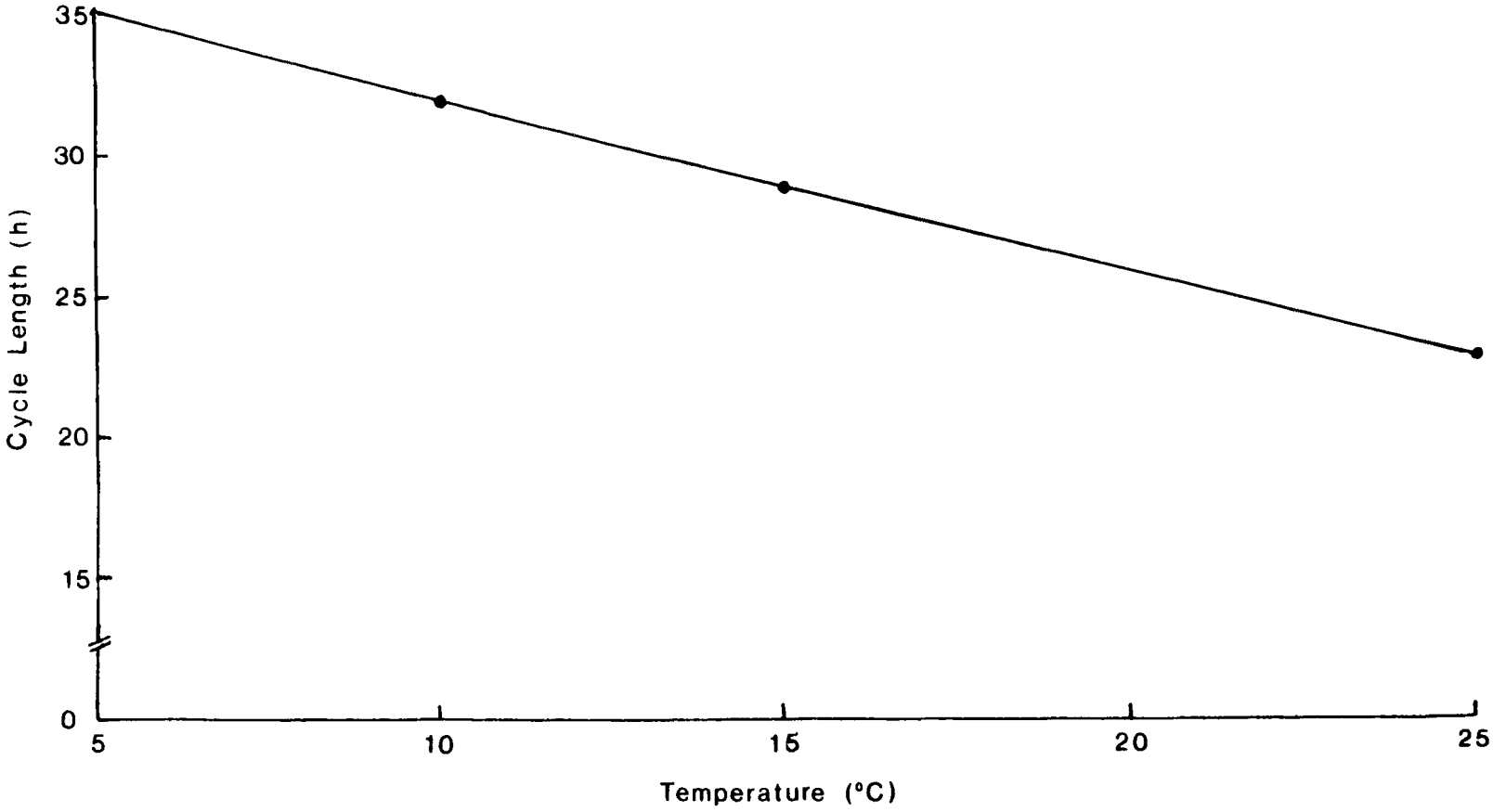


Fig. 2.1 Optimal Cycle Length at Different Temperatures for Saintpaulia ionantha. (After F W Went, 1960).

time switches (Sauter memotime, time switch type 25 D7). The time switches were programmed weekly for the required extended cycle lengths which were: 16h light/16h dark, 10°C and 14h 30min light/14h 30min dark at 15°C.

2.11 Growth Analysis

2.11.1 Tissue Cultures

In all experiments the explants were observed, using a low powered (x30 magnification) Vickers bench binocular microscope, at intervals of either 7 or 28d for periods up to 6 months. The frequency of explants producing callus (explant response), the callus area and the number of shoots and roots produced by the responding explant (explant productivity) were tabulated along with fresh and dry weights. Also qualitative observations of the changes in callus colour, texture and form were documented.

2.11.2 Characterisation of Plants

All stock, anther-derived and selected plant lines were evaluated. The following characteristics were measured: plant diameter; leaf number; leaf length; leaf width; leaf form; number of flower stalks and flowers; flower length; flower width; and flower form.

2.11.3 Guard-cell Chloroplast Determination

Chloroplast counts were made from the stomatal guard cells in anther-derived and stock plants. Using fine forceps epidermal strips were taken from the underside of a young and mature African violet leaf and placed in a drop of distilled water. After the coverslip was placed over the peel, the slide was observed under high magnification of a

light microscope. For each leaf a sample of 25 stomates were scanned and the number of chloroplasts present was recorded.

2.11.4 Karyotypic Analysis

Chromosome counts were made from root-tips of anther derived and stock plants. Root tips were prepared by using a modified Feulgen staining procedure devised by Roberts and Short (1979). Young roots, less than 5mm long, were taken from three-week-old leaf cuttings grown in Hoagland's solution. The roots were then immersed in 0.1 per cent colchicine solution for five hours at room temperature. After washing the root tips in two changes of distilled water the root tips were fixed in freshly prepared acetic alcohol (glacial acetic acid: absolute ethanol, 1:3 v/v), and left overnight at 4°C. The fixed material was then washed in two changes of distilled water prior to being hydrolysed in 3.5M HCl at 37°C for 20 minutes (the acid was preheated to this temperature before the root tips were introduced). After a further wash in three changes of distilled water the root tips were immersed in Feulgen reagent for three to four hours. The stained root tips were then washed twice in distilled water prior to immersion in 45% glacial acetic acid for a least 30 minutes (up to two days). Slides were prepared by placing an excised root on a clean microscope slide and discarding all but the terminal 2mm. The tip was fragmented with a scalpel blade, two drops of 45% glacial acetic acid were added then a coverslip was placed over the preparation. Without applying pressure, one end of the coverslip was held down with filter paper and the coverslip was gently tapped with a blunt instrument to disperse the cells. The preparation was gently warmed over a spirit lamp. While the slide was still warm, pressure was applied with a thumb to the coverslip, through several layers of filter paper, ensuring that there was no lateral

movement. The preparation was checked at this stage to ensure that it was of suitable quality. If a permanent preparation was required a razor blade was inserted under one corner of the coverslip and the coverslip was flicked off in one movement. Some cells remained attached to the slide and others to the coverslip. Both slide and coverslip were rapdily transferred to absolute ethanol in a staining trough and left for at least three minutes. The coverslip was mounted in Euparal (Hopkin and Williams Ltd) on a clean slide. Euparal was added to the prepared slide before a coverslip was placed over the stained root material A fragment of filter paper was placed at the edge of the coverslip to absorb surplus fluid and prevent it from sliding off.

For quick screening of plant karyotypes the rapid HCl/toluidine blue squash technic for plant chromosomes was used (Marks, 1973). The reagents required were 5N HCl and a 0.05% solution of toluidine blue stain (G T Gurr's, 04800) buffered at pH 4. The stain was made up in McIlvaine buffer (0.1M Citiric acid, 0.2M Na_2HPO_4). Root tips were either pretreated in 0.1% solution of colchicine or used directly from the plant. The root matrial was then hydrolysed in 5N HCl for 15 min at room temperature. After rinsing in distilled water slides were made immediately, or if necessary, roots could be stored in distilled water at $4^{o}C$ for up to 24 hours without deterioration. The root tip is then macerated with a needle in a drop of stain on the slide. Maceration was continued until the tissues were broken up into small, well-stained fragements. The preparation was completed by adding a coverslip and applying gentle finger pressure through a double thickness of filter paper.

All preparations were examined by scanning the slides systematically and recording the number of chromosomes present in a representative sample of cells.

2.12 Histological Preparation

Small tissue explants were fixed in formalin aceto-alcohol (90ml, 70% ethanol, 5ml, glacial acetic acid and 5ml 40% formaldehyde) for at least 12h. Following fixation the tissues were placed in compartmentalized metal mesh containers and washed in 50% ethanol for 1½h. The containers were then transferred to the histoclinet (Shandon-Elliot, Automatic Tissue Processor), to be dehydrated, cleared and embedded. Explants were dehydrated through a graded series of ethanol solutions for varying amounts of time (50%, 3h; 70%, 3h; 90%, 3h; 100% I, 1h; 100% II, 1h; 100% III, 1½h; 100% IV, 1½h). Tissues were then cleared in 3 changes of toluene (1½h, 2h and 2h) before being infiltrated with molton wax 3 times (at 1½h intervals). Specimens were blocked out in molten wax and placed in the freezing compartment of a refrigerator in preparation for sectioning.

Sections were cut (3-7 μm) using a Leitz Wetzlar 1512 Rotary microtome and transferred onto a slide thinly smeared with albumin. The slide was flooded gently with distilled water, so the sections floated, and then placed on a hot plate at a temperature a little below the melting point of wax. The sections extended as they warmed up. then they were fully stretched they were arranged in their final postions and the excess water was drained off. The sections were then dried overnight at about 30°C. Preparations were stained with 0.05% (w/v) aqueous toluidine blue for 5-10 min and warmed on a hot plate at 30°C. Excess stain was washed off by immersing the slide in 70% ethanol and then distilled water for 5 min each. Sections were air dried for one week before being made permanent by mounting in Euparal.

2.13 Scanning Electron Microscopy

Anther explants were fixed in a 3% glutaraldehyde (made up in 0.1M phosphate buffer at pH7) solution for

24-48 hrs. After fixation the anthers were rinsed in the buffer solution for ½h then dehydrated through a graded series of increasing acetone concentrations (30%, 50%, 70%, 90%, 100% (two changes) requiring at least 2h at each step. All fixation washing and dehydration was carried out at 4°C.

Following dehydration the specimens are critical point dried, using CO_2 as the transitional fluid, in a Polaron critical point drying apparatus (Watford Ltd).

Dried anthers were coated with 15nm of gold using a Polaron sputter coating apparatus (Watford Ltd)

All specimens were observed with a JEOL Ltd T200 scanning electron microscope using collected secondary electrons.

2.14 Electrolyte Leakage

Exposure of plant material to low temperature often results in the disruption of cell membranes with a consequential release of electrolytes. Thus a measure of the electrolyte leakage from plant material provides a rapid method for indicating the cold tolerance of tissues.

Isolated 1cm leaf-discs (5 discs per 9cm Petri dish) were incubated in 25ml deionized water in the dark at temperatures of 5°, 10° and 25°C. Afer 24h the ion solution was decanted, from each of the twelve replicates, into 50ml beakers and a conductivity reading was taken (Model P335 Portland Electronics Ltd, Oldham). Ion solution and leaf-discs were then boiled in McCartney universals for one hour. After cooling the solution to room temperature another conductivity reading was taken to measure the total electrolyte leakage. Using the above two sets of values percentage electrolyte leakage was calculated.

Deionized water controls were prepared and treated in the same manner. The experiment was repeated three times (36 replicates) and the results were combined before analysis.

2.15 Mutagenesis

Warfield (1973) reported that treating Saintpaulia petioles with Ethyl-methane-sulphonate (EMS) induced mutations in the plantlets produced. Therefore in this study attempts were made to induce somatic mutations using EMS.

To determine the correct mutagen dosage surface sterilized leaf-discs were exposed to 0, 1, 2, 3, 4, 5 and 10% levels of EMS for ½h. The discs were then rinsed four times in sterile distilled water and plated upper surface face down, on 25ml of MS regenerative media (5 discs per 9cm Petri dish). EMS concentrations were prepared by pipetting the appropriate aliquot into 0.01M phospate buffer. This solution was filter sterilized using 0.22 um nitrocellulose membrane filters (nucleopore Corp, Pleasanton, California, USA). The above method was used in all mutagenesis experiements carried out. All experiments were designed to include the appropriate phosphate buffer controls.

CHAPTER THREE

POLLEN DEVELOPMENT, ANTHER AND POLLEN CULTURE OF AFRICAN VIOLET

3.1 Introduction

Studies on anther culture show that success depends largely on the developmental stage of the pollen within anthers inoculated onto the culture media. This may vary with the species and possibly with the cultivar (Sunderland and Dunwell, 1977). Previous studies with African violet indicates that the optimum stage for pollen anther culture is when the pollen cells are in or near the first pollen mitosis (Hughes, et al, 1975; Weatherhead et al, 1982).

The correct stage of anther development for haploid induction can be determined accurately by the time-consuming direct cytological examination of the pollen within anthers of individual flower buds. However, in practice, it is convenient to resort to some external morphological feature, such as; bud length, bud diameter, corrolla length and the length of the emerging flag leaf or spike, for selecting anthers at the right stage prior to culturing. Work of Hughes et al (1975) indicates that anthers from buds 3-5mm in diameter produced the highest number of plantlets. Anthers from buds smaller or larger produced neither callus nor plantlets (Chapter 1, section 1.9).

One of the new emerging concepts in anther and pollen culture studies is the phenomenon of pollen dimorphism in relation to pollen plant formation (Chapter 1, section 1.6.2.). Pollen dimorphism refers to the presence, in an anther, of pollen of different morphological and staining characteristics which have the potential to develop into two different types of generations - gametophyte and sporophyte. Evidence from the anther culture of barley (Dale, 1975) and tobacco (Horner and Street, 1978) has shown a correlation between the

frequency of dimorphic pollen *in situ* and the formation of embryos on culture of anthers. Furthermore, the above authors found that pollen capable of forming embryos are different from those destined to form gamates, and can be distinguished in anthers prior to culture. The embryogenic pollen are small, and have a cytoplasm that stains less intensely with acetocarmine than the gametophytic (non embryogenic pollen.

Previous investigations have shown that various physiological, nutritional and physical factors can influence the induction and production of plantlets from anthers (Chapter 1, section 1.6.4). These include the growing temperatures of the donor plant. (Primo-Millo and Sunderland, 1976; Thurling and Chay, 1984), chilling pretreatment of excised flower buds prior to culturing (Nitsch and Norreel, 1973; Rashid, 1982), incubation temperatures of anther cultures (Keller and Armstrong, 1981) and the effect of light/darkness on haploid production (Sunderland, 1980; Hidaka, 1984).

Furthermore, the composition of the medium is one of the most important factors determining not only the success of anther culture but also the mode of development (Chapter 1, section 1.6.4.3). In recent years a number of anther culture studies have been carried out with *Saintpaulia ionantha* in the attempt to produce haploid plants. (Hughes et al, 1975; Smith, et al, 1981: Norris, et al, 1982; Weatherhead, et al, and Bhaskaran, et al, 1983) (Chapter 1, section 1.9). Hughes, et al (1975) and Smith, et al (1981) described low levels of haploid production, typically 1-5 plantlets per anther from 3-18% of anthers cultured on a single defined medium (Blaydes, 1966). However, Weatherhead, et al (1982) investigating a range of culture media found that up to 31% of anthers cultured on solidified MS medium supplemented with NAA:BAP at 1:0.5mgl^{-1} (thereafter designated as the medium of Weatherhead, et al) produced plantlets (Chapter 1, section 1.9).

Further alterations in the compostion of the culture medium have been shown to improve the anther response to culture (Vasil, 1980). Previous research has found that changes in levels of sucrose (Keller, et al, 1975), growth regulators, particularly auxins and cytokinins (Lichter, 1981; Chen, et al, 1982), Iron (Vagera and Jilek, 1984) and the addition of activated charcoal (Anagnostakis, 1974; Johansson, 1983) and Polyvinyl polypyrrolidone (Tyagi, et al, 1981) can increase production from anthers.

Over the last fifteen years new techniques have been devised to culture isolated pollen. Kameya and Hinata (1970) used a hanging drop method to culture isolated pollen of Brassica oleracea and B oleracea x B alboglabra. Their method involved placing a drop of medium containing 50-80 grains on the cover glass, which is then inverted over a cavity slide and sealed with paraffin. More recently Sunderland and Roberts (1977) introduced another method to produce embyoids from pollen grains, which was developed for Nicotiana tabacum. This method requires the culture of anthers in liquid medium where they dehisce and shed pollen into the medium. By transferring the anthers to fresh medium at intervals, a series of cultures of 'free' pollen is obtained which develop into embryoids. Also Sharpe, et al (1972) induced the pollen of Lycopersicon esculentum to form haploid callus by using a nurse culture technique.

3.2 Aims and Experimentation

The aims of this study were to induce, and optimise haploid plant production from anthers and pollen of African violets. Thereafter plant lines were to be used in mutation studies to screen for low temperature tolerance (Chapters 5 and 6).

In order to satisfy the above aim it was necessary, primarily, to investigate the development of African violet pollen from pollen mother cell to anthesis to correlate between these stages and an external morphological marker. Furthermore, an assessment was undertaken to evaluate pollen dimorphism in Saintpaulia anthers prior to culture. To investigate pollen ontogeny and dimorphism a representative sample of anthers was taken from a series of young to old buds of known diameter). Anthers were then squashed in acetocarmine and examined under high power using a light microscope to determine their stage of development (Chapter 2, sections 2.4.2 and 2.4.3).

To determine the influence of culture medium on callus and shoot production from African violet anthers two media were compared. Anthers at different stages of development were cultured on the media of Blaydes (1966) and Weatherhead, et al (1982). Cultures were incubated at 25^{o}C under a light regime of 16h light and 8h dark. (Chapter 2, section 2.4.5). After 28 and 56d the frequency of anthers producing callus, and the number of shoots and roots produced per explant were determined (Chapter 2, section 2.11.1). Thereafter by using the most responsive medium the growth and development of African violet anthers in culture was assessed over a period of 56d by quantitative and qualitative means.

To assess the effect of anther culture on the frequency of viable microspores/pollen a sample of anthers, from each of the five bud stages, were selected after 7, 14 and 28d of culture and squashed in fluorescein diacetate. One hundred grains were scored for the presence of viable (yellow/green) and non viable (dark green) microspores or pollen grains (Chapter 2, section 2.4.4).

An investigation was undertaken to determine the origin, growth and subsequent development of in-vitro produced African violet anther callus. This was achieved with the aid of light as well as scanning electron microscopy. Callus and shoot production was induced by culturing anthers at different stages of development on the most responsive medium. Anthers were sampled at 21, 28 and 42d and processed for microscopy as described in Chapter 2 (sections 2.12 and 2.13). Furthermore, experiments were carried out to determine, at what frequency isolated anther wall connective/pollen and filament tissues produced callus, when cultured on the most responsive medium.

Various physiological, physical and nutritional factors were investigated to test whether they enhanced callus induction and shoot production from in-vitro African violet anthers. For the following series of experiments anthers were always cultured on the most responsive medium.

To test whether the temperature under which donor plants are grown influences the development of anthers in culture African violet plants were raised in growth cabinets at 15°, 20° and 25°C. Following harvest anthers were cultured at 25°C with a 16h photoperiod. After 28 and 56d anthers wereassessed for callus, shoot and root induction (Chapter 2, section 2.11.1).

An investigation was undertaken to evaluate the influence of low temperature bud pretreatment on callus induction from cultured African violet anthers. Excised buds were maintained in the darkness at 8°C for 7 or 14d (Chapter 2, section 2.4.1). Anthers were cultured at 25°C, and assessed after 28d for callus induction. Furthermore, to determine the effect of culture temperature on the frequency of callus induction and subsequent growth and

development, anthers were incubated at 10°, 15°, 15°(16h)/25°(8h) and 25° with a 16h day length. Callus induction was assessed at 14, 28 and 56d. Cultures were harvested after 56d and callus growth, shoot and root production was determined.

Anthers of different stages were incubated in the dark at 25°C to test whether such a treatment was beneficial to callus induction.

To investigate the influence of the composition of the culture medium on callus induction and shoot and root production from cultured anthers, various components within the medium were altered. In order to determine which concentrations of sucrose would be optimal in the medium, different amounts of sucrose (3 to 15%) were tested. Similarly Auxin and Cytokinin levels were optimised by culturing anthers on medium containing NAA:BAP at concentrations of 1:0.5, 1:1 and 0.5:1 mgl^{-1} respectively. Furthermore, the addition or the absence of Iron ions, activated charcoal and Polyvinylpolypyrrolidone were investigated to determine whether such components were beneficial to callus induction. As before anthers were cultured in the light at 25°C (with a 16h photoperiod) for periods up to 56d. Callus induction was determined after 28d and shoot and root production at the 56d harvest.

To investigate whether culturing African violet pollen, in various concentrations and combinations of MS medium and sucrose would cause division, hanging drop cultures were prepared as described in Chapter 2 (section 2.5.1). The concentrations of MS medium tested were 100, 10 and 1% . Sucrose was added to the three concentrations of MS medium at levels of 3, 6, 10, 12 and 15%. Cultures were incubated for 56d at 25°C either in the light (with a 16h photoperiod) or in the dark.

To determine whether low temperarure pretreatment of buds is beneficial to the frequency of dehishence, anthers at different stages of development were incubated at 8°C for 8d then cultured on liquid A medium (Nitsch, 1974) and incubated at 25°C in the darkness. After 14d, cultures were assessed for the frequency of dehiscing anthers. Consequently, a series of experiments were undertaken to evaluate the influence of temperature pretreatments on the induction frequency of microspore/pollen division. African violet buds were pretreated at 4, 8, 15, 20, 25 and 25°C for varying lengths of time ranging from 0-28d. Thereafter anthers were excised and cultured on liquid A or AGSI medium (Nitsch, 1974) and incubated at 28°C in the darkness for the first 14d (Chapter 2, section 2.5.2). After this time cultures were transferred to the light (16h photoperiod) and assessed at 28 and 56d for dividing pollen.

Pollen was isolated from anthers and cultured on an anther nurse tissue to determine whether such a treatment would induce pollen division (Chapter 2, section 2.5.3). All cultures were incubated at 25°C in the light (with a 16h photoperiod) and the experiments were assessed at 28 and 42d.

3.3 Results

3.3.1 Pollen Development

Cytological observation of pollen development was done with freshly harvested flower buds. In all, 10 anthers were examined giving a total of ca100 pollen grains.

The following stages were observed: pollen mother cells (PMC) at all steps of meiotic division I (prophase, metaphase, anaphase and telophase),

tetrads, uninucleate and binucleate pollen grains (Fig 3.1). The PMCs are thin-walled and oblong (Fig 3.1a). The undergo division to give rise to lenticular shaped microspore tetrads with a thick callose wall (Fig 3.1b,c). PMCs in meiosis and tetrads were predominant in buds of breadth 2.3$\pm$ 0.4 and 3.4$\pm$ 0.2mm respectively (Table 3.1). Wall formation of tetrads started before formation of the nuclear membrane and reorganisation of the daughter nuclei (Fig 3.1c).

Tetrads were released from the mother-cell wall as thin-walled, more or less spherical calls. (Fig 3.1d). These enlarged rapidly, mainly by vacuolation (Table 3.2) each became invested with a tough outer coat (exine) and a inner wall (intine) (Fig 3.1 e,f).As the vacuole expanded the nucleus was pushed close to the spore wall near to one end of the spore (Fig 3.1e). In this position, the nucleus underwentreplication and increased in size prior to entering the first pollen mitosis (Fig 3.1f).

The development of uninucleate microspores fromt he time of their release from the original PMCs until the first mitosis occured in buds ca. 5.5$\pm$ 0.4mm in diamter

Pollen scored from buds 7.4$\pm$ 0.4mm in diameter were characterised by an asymetrical microspore division resulting in a small generative nucleus and a large vegetative nucleus. The generative nucleus stained slightly deeper with acetocarmine compared to the vegetative nuclues (Fig 3.1g). During the final phase of maturation, the pollen grains enlarged further (Table 3.1) and large amounts of starch accumulated in the plastids prior to anthesis (Fig 3.1h).

Fig. 3.1 Stages of pollen development in Saintpaulia ionantha. a & b: Pollen mother-cell at meiotic pro-, meta-, ana- and telophase I (stage 1 anther). c & d: non-vacuolate tetrads before and after release from the mother-cell wall (CW) (Stage 2 anther). e: Vacuolate uninucleate spore before DNA replication (Stage 3 anther). f: Haploid spore in anaphase of the first pollen division (Stage 4 anther). g & h: Binicleate pollen grains before and after deposition of starch. Note the small generative nucleus (G) distant from the intine and the larger vegetative nucleus (V). The vacuole is completely resorbed (Stage 5 anther). Calibration bar = 10µm.

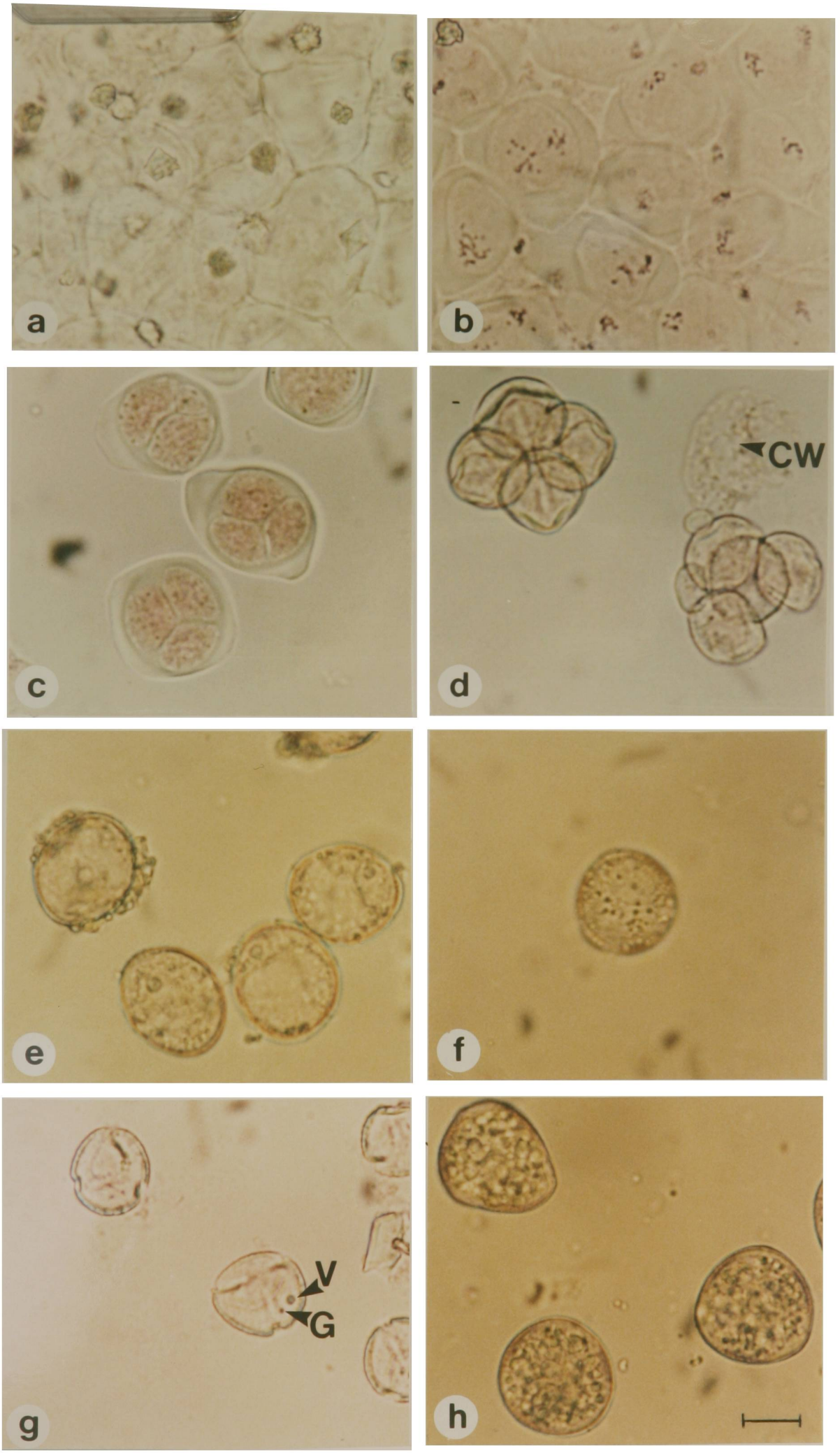

a
b
c
d
CW
e
f
g
V
G
h

Table 3.1 Pollen Dimorphism in Saintpaulia ionantha. The Frequency of Normal Pollen and Potential Embryogenic Pollen with their Respective Pollen Diameters

	Normal Pollen	Potential Embryogenic Pollen
Grain Type	62.4±5.9	37.6±5.9
Pollen Diameter (um)	20.7±0.5	16.5±0.3

One anther from each of 10 stage 5 buds sampled. Anthers crushed in Acetocarmine. Hundred pollen grains scored per anther. Mean and standard errors are indicated.

Table 3.2 Saintpaulia ionantha cv 'Blue Rhapsody'. Correlation Between Stage of Pollen Development and Bud Diameter

Stage	Mean Bud diameter (mm)	Mean Pollen diameter (um)	Distribution of Grain-Types (%)					
			M	T	U	Mi	Bs	DE
1	2.3±0.4	13.4±0.4	100±5.5	-	-	-	-	-
2	3.4±0.2	15.9±0.4	11.3±2.2	88.7±0.8	-	-	-	-
3	5.5±0.4	19.5±0.8	-	9.4±4.7	90.6±5.8	-	-	-
4	7.4±0.4	20.9±0.6	-	-	-	96.1±1.5	3.9±0.6	-
5	8.6±0.5	22.1±0.3	-	-	-	43.5±4.5	55.7±2.5	0.8±0.8

Buds were harvested from inflorescences of ten Blue Rhapsody plants. Buds of similar diameter were grouped together. For each bud group one anther was selected from ten buds and squashed in Acetocarmine. Hundred pollen grains were scored per anther. Standard errors are indicated. M = mother cells in meiosis; T = tetrads; U = Uninucleate pollen grains; Mi = grains undergoing first mitosis; Bs - binucleate graisn with starch; DE = degenerating or empty grains.

3.3.2 Pollen Dimorphism in Mature Pollen Grains

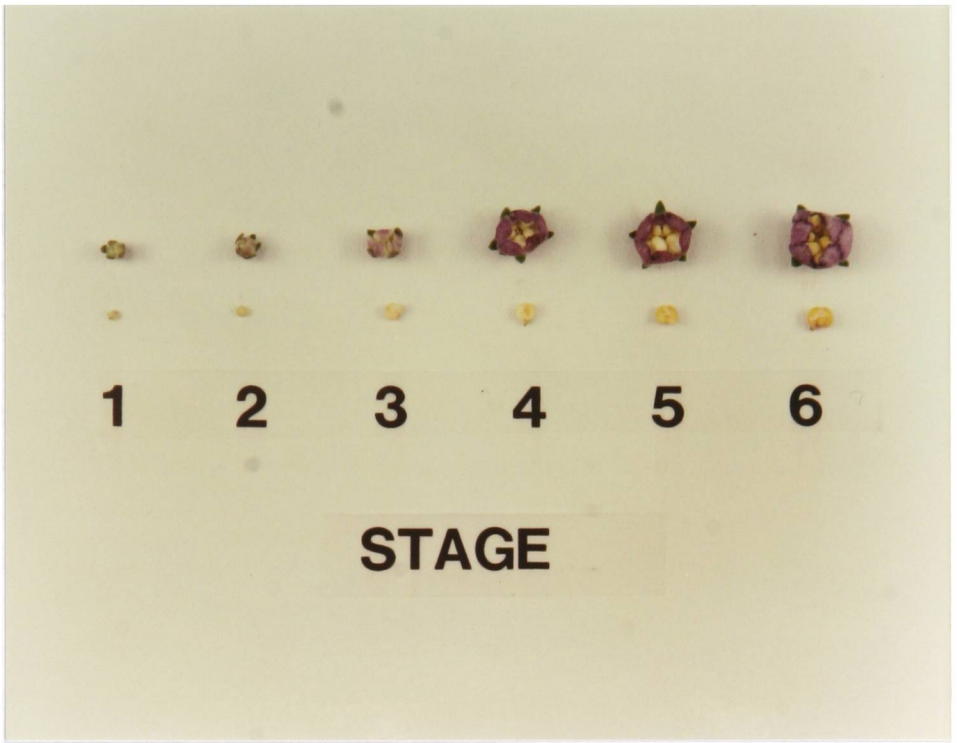
During routine examination of pollen grains from mature anthers (excised from buds 8.6±0.5mm in diameter) a dimorphism was observed. The majority of grains (designated as normal grains) were 20.7± 0.5μm in diameter, contained deposits of starch and their cytoplasm stained so deeply with acetocarmine that the nuclei were often obscured (Table 3.1 and Fig 3.2). However, nearly 40% of pollen grains were smaller (16.5±0.3μm) contained no discernible starch and their cytoplasm stained very weakly in acetocarmine. Such grains were identified as potential embryogenic pollen.

3.3.3. Staging of Anthers

The relationship between stage of pollen development and bud diameter is represented in Table 3.2. Observations showed that there was little variation in stage of pollen development amongst flower buds taken from the same portion of different inflorescences. However, sample buds from different portions of the same inflorescence exhibited different degrees of pollen maturity. Upper flowers were more mature than basal ones. However, as the development of the pollen in an anther was not always synchoronous, the stage of an anther was defined according to the stage of majority of the pollen in the anther. Five stages were distinguished as follows (Fig 3.3a):-

1. Pollen mother cell undergoing meiosis
2. Tetrad stage of meiosis
3. Uninucleate microspores
4. Microspores at mitosis
5. Binucleate stage

Fig. 3.2 Large densely stained (aceto-carmine) normal pollen with starch grains and smaller light staining potential-embryogenic pollen grains. Bar = 10μm.



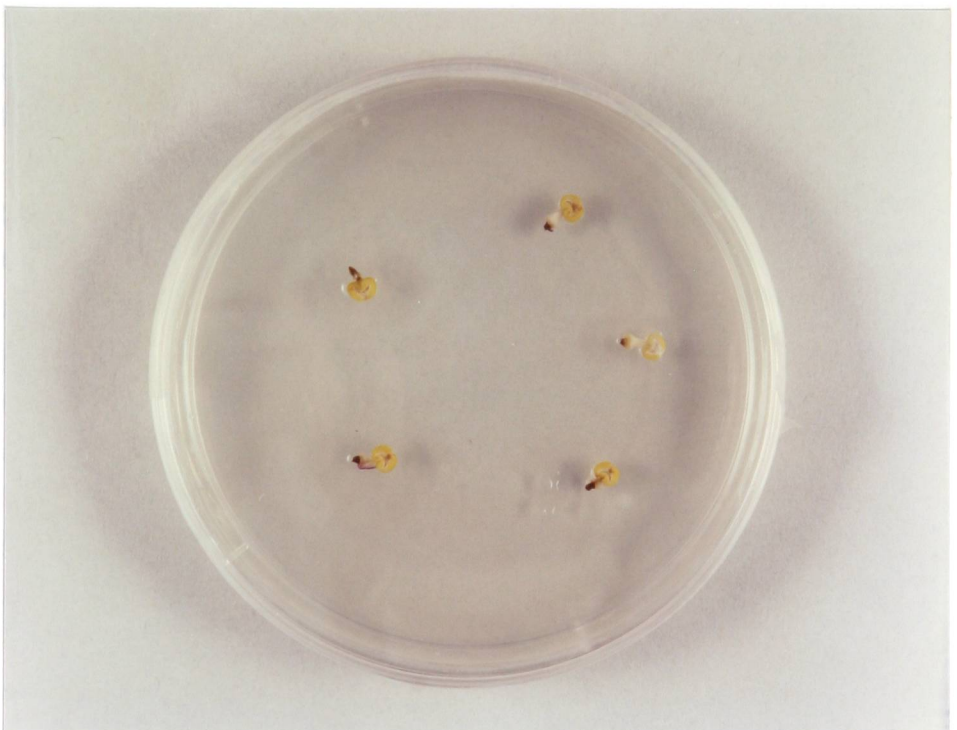


Fig. 3.3.a: The five developmental stages of the buds with corresponding anthers (Natural size).

b: Anther culture of Saintpaulia ionantha (Stage 5 anthers. Natural size).

3.3.4 Influence of Culture Medium on In-Vitro Culture of African Violet Anthers

Callus production from within anthers was observed on both the media of Blaydes's (1966) and that of Weatherhead, et al (1982). After 4 weeks in culture optimum callus induction was observed from anthers excised from the older bud stages and cultured on the medium of Weatherhead, et al (Fig 3.4). About 63% of stage 5 anthers cultured on the medium of Weatherhead, et al (1982) induced callus compared to 23% cultured on Blaydes's medium. Overall, callus induction on Blaydes's medium was exceptionally poor. As little as 5% of stage 3 anthers produced callus and no callus was induced from stage 1 and 2 anthers cultured on Blaydes's medium (Fig 3.4). The above results were reflected the data obtained for the induction and production of shoots and roots (Tables 3.3 and 3.4). The results in Table .3 show that whereas only 0.9% of stage 3 anthers plated on Blaydes medium induced both shoots and roots more than 48% did so on the medium of Weatherhead, et al (1982). Furthermore a maximum of 32.4 shoots were produced from stage 5 anthers after 8 weeks culture on the latter medium; the younger anther stages producing progressively less shoots (Table 3.4). As the medium of Weatherhead, et al (1982) was more effective at inducing callus, shoots and roots from African violet anthers, compared to Blaydes's medium, it was therefore used in further studies undertaken to optimise shoot production from Saintpaulia anthers.

3.3.5 Development of Anthers in Culture

Preliminary experiments (Table 3.5) indicated that the frequency of callus induction on the medium of Weatherhead, et al (1982) was doubled if anthers were cultured with filaments (intact). Prior to further experimentation a developmental sequence

Table 3.3 Organogenesis in African Violet Anthers Cultured on the Media of Blaydes (1966) and Weatherhead, et al (1982)

	Blaydes Medium			Medium of Weatherhead, et al		
Bud Stage	Shoot Only	Root Only	Shoot & Root	Shoot Only	Root Only	Shoot & Root
1	0	0	0	0	8.1 ± 2	12.2 ± 3.7
2	0	0	0	16.7 ± 3.2	0	16.7 ± 7.1
3	2.7 ± 0.8	0	0.9 ± 0.5	9.6 ± 1.9	3.2 ± 1.9	48.4 ± 3.2
4	0.9 ± 0.9	0	0	2.5 ± 2.3	12.8 ± 3.5	43.6 ± 4.9
5	1.8 ± 1.2	0	0.9 ± 0.5	8.6 ± 1.0	6.5 ± 2.7	39.1 ± 5.6

Frequency of shoot and root differentiation (%). Anthers cultured for 56d on the media of Blaydes (1966) and Weatherhead, et al (1982). Sucrose concentration was 3%. Cultures incubated at 25°C under a period of 16h light followed by 8h darkness. A minimum of 50 anthers were used for each treatment. All treatments were duplicated. Standard errors are indicated.

Table 3.4 Effect of the Media of Blaydes (1966) and Weatherhead, et al (1982) on Shoot and Root Production in Cultured Anthers of African Violet

	Blaydes Medium		Medium of Weatherhead et at (1982)	
Bud Stage	Shoot No	Root No	Shoot No	Root No
1	0	0	6 ± 1.3	5.7 ± 2.5
2	0	0	8.5 ± 2	9.0 ± 1.4
3	2.2 ± 1.1	2.2 ± 1.1	15.6 ± 2.9	8.1 ± 1.5
4	1.1 ± 0.8	0	25.6 ± 5.3	7.1 ± 1.8
5	4.8 ± 0.9	2.2 ± 1.1	29.1 ± 3.3	12.4 ± 0.3

Mean numbers of shoots produced per explant after 56d culture on the media of Blaydes (1966) and Weatherhead, et al (1982). Sucrose concentration was 3%. Cultures incubated at 25°C with a 16h photoperiod. A minimum of 50 replicates were used for each treatment. All treatments were duplicated. Standard errors aare indicated. Plantlets derived from anthers cultured on Blaydes medium were regenerated and transferred to compost to be characterised after six months (Chapter 4).

Table 3.5 Callus Induction Frequency of In-Vitro Cultured African Violet Anthers

Induction Frequency of Callus (%)	
Anthers Cultured Without Filament	Anthers Cultured Intact
31.4±7.5	68.6±7.5

Anthers cultured with or without filament for 28d on the medium of Weatherhead, et al (1982). Cultures incubated at 25°C with a 16h photoperiod. n = 50. Both treatments were duplicated. Standard errors are indicated.

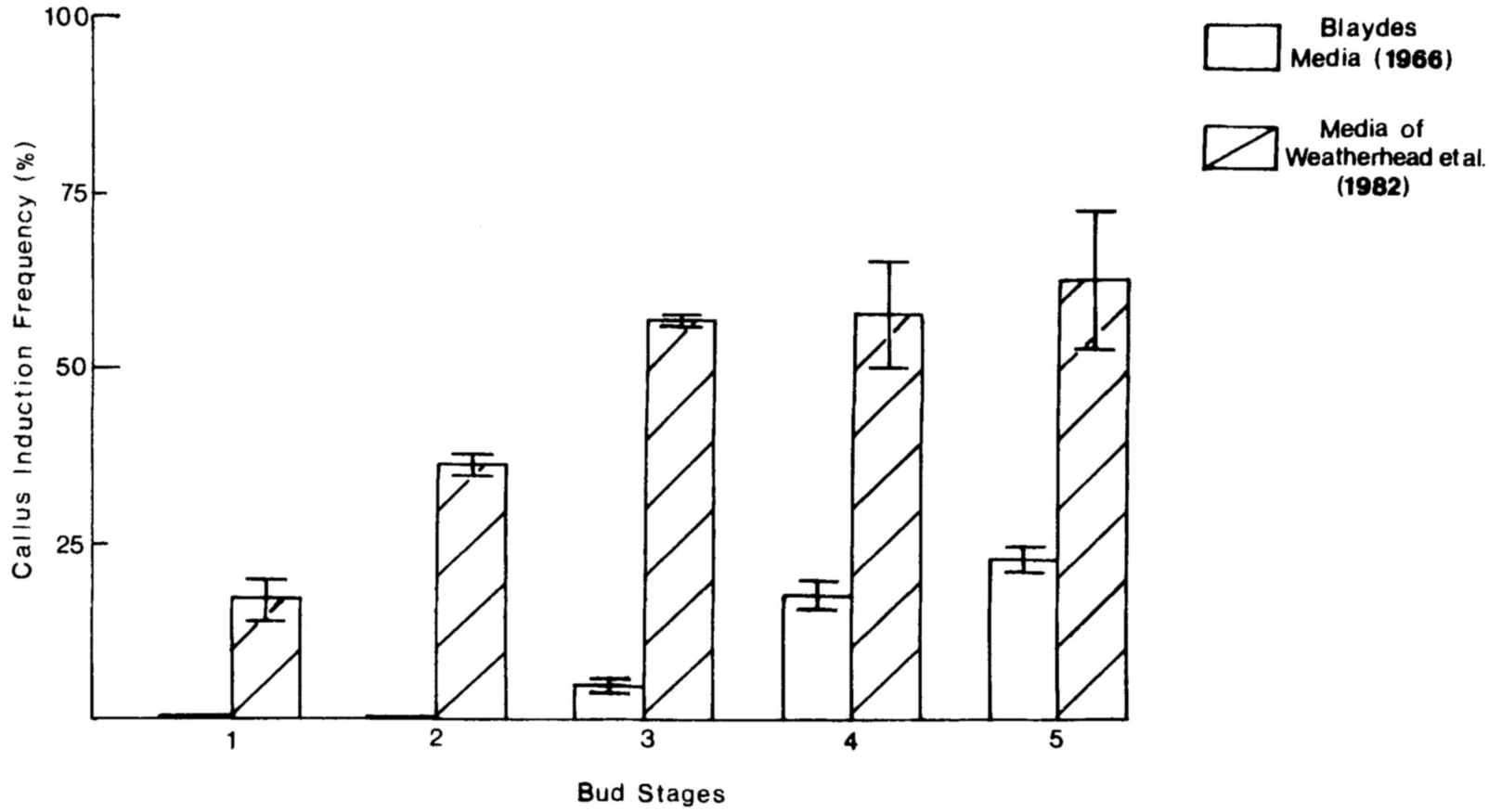


Fig. 3.4 Influence of Culture Medium on Callus Induction. Culture period of 28d. One hundred anthers used for each bud stage. All treatments were duplicated. Standard errors are indicated.

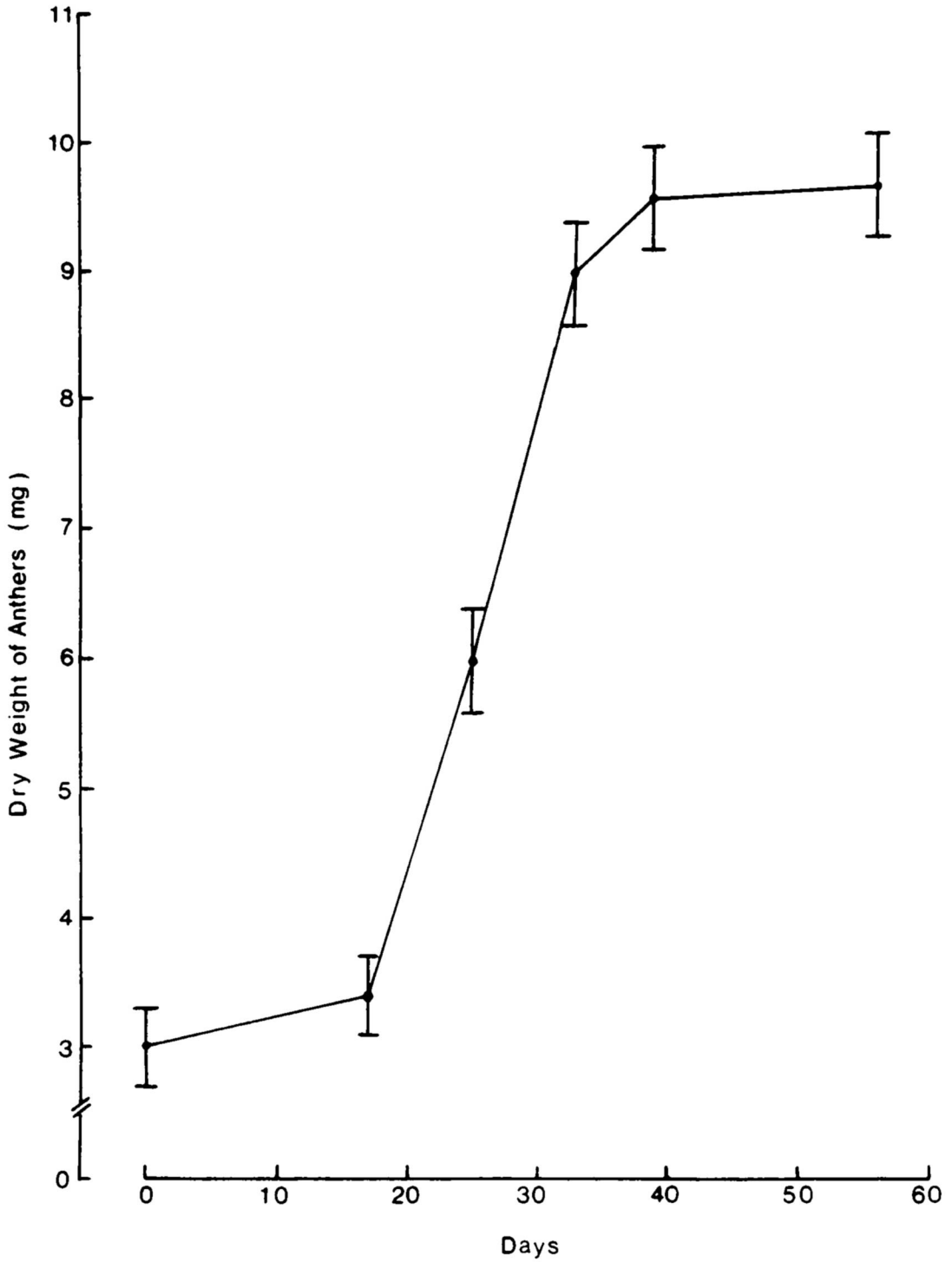


Fig. 3.5 Growth of <u>In-vitro</u> Cultured Anthers. Stage 3 anthers cultured on the medium of Weatherhead, et al (1982) and incubated at 25°C under a 16h photoperiod. n = 30. Standard errors are indicated.

for cultured intact African violet anthers was determined as described below.

3.3.5.1 Callus Initiation and Differentiation

After 7d of culture, on the above medium, anthers became visibly swollen (Fig 3.6a) and callus production from within the anther tissues commenced soon after (Table 3.6 and Fig 3.6b). Within 28d the frequency of anthers producing callus increased to a maximum of 60%. This high response was confined to cultures, which had been initiated with anthers excised from the older bud stages. The callus was creamy-brown in colour and grew as a compact globular mass showing a 3-fold increase in dry weight over a 5-week growth period (Fig 3.5). When maintained on the medium under a regime of 16h light per day callus organised, at the surface, into nodular aggregates of cells (Fig 3.6 c) that subseuqnetly differentiated into shoot or root primordia. Within 6 weeks many aggregates rapidly developed green colouration, and shoot primordia with glandular hairs developed on them (Fig 3.6d and Table 3.6). After a further two weeks incubation, small leaflets already showing leaf hairs and the basic external morphology of the Saint paulia leaf were soon apparent (Fig 3.6e). These leaflets underwent expansion and subsequent petiole extensions, and discrete plantlets developed which could be dissected from the callus surface and transferred to rooting-medium. Within 8 weeks of culture (Table 3.6) the frequency of anther explants regenerating plantlets ranged from 15% (bud stage 1) to just under 40% (bud stage 4). This response might have been higher were it not for the high percentage of collapsed or degenerating anthers observed among those excised from older buds. Also, the tissue derived from the anther frequently produced a slow growing, dark brown callus that did not differentiate under those growth conditions (Table 3.6). Cultures

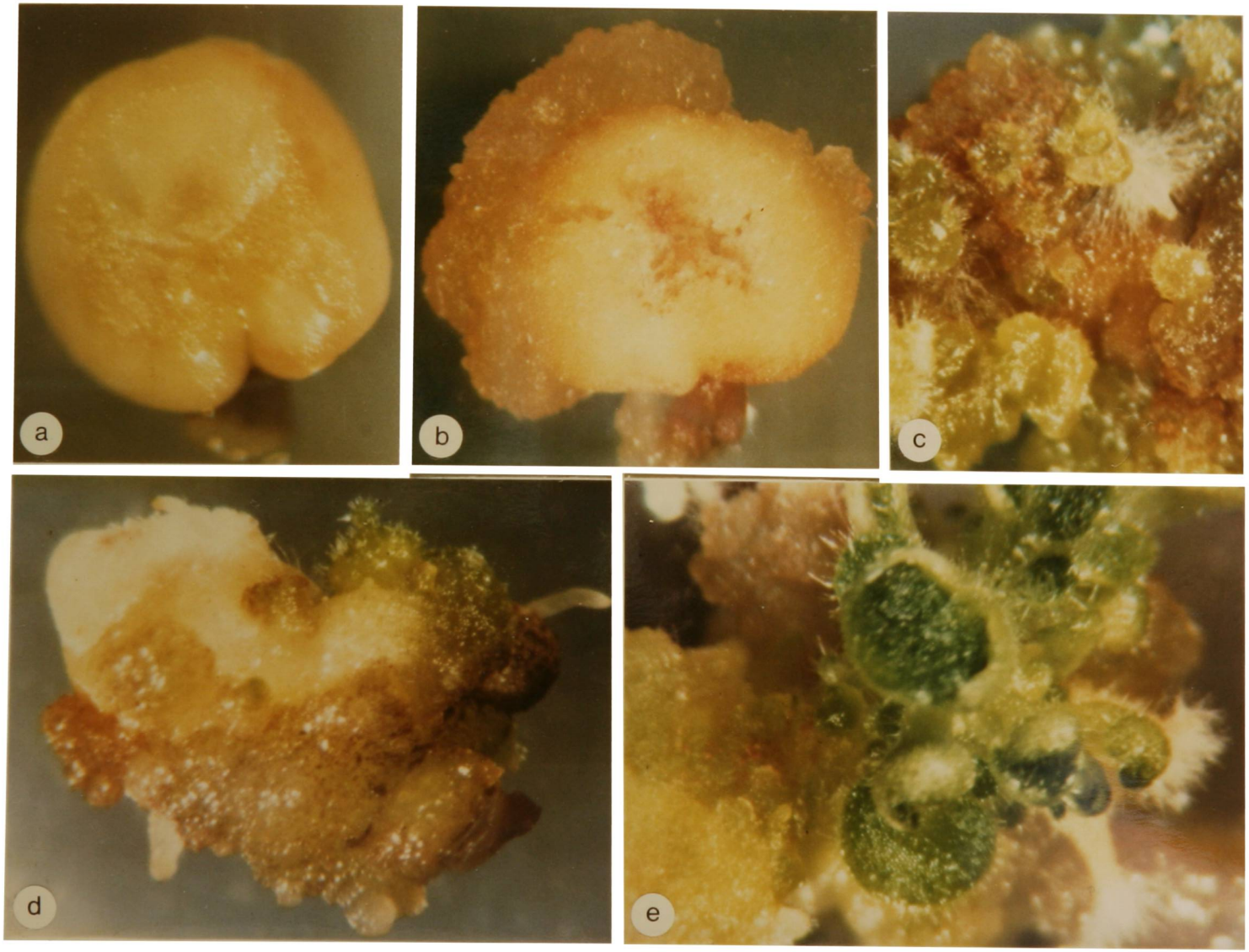

a
b
c
d
e

Table 3.6 Development of Cultured Saintpaulia ionantha Anthers

Days of Culture	Bud Stages	Stages in Anther Development (%) W	Y	CBC	DBC	GN	S	R	S & R	C
	1	60	40							
	2	30	70							
7d	3	33.4	66.6							
	4	24	76							
	5	25	75							
	1	42	58							
	2	30	57	3						
14d	3	29.4	64.6	6						
	4	23	72	5						4
	5	12	54	9						25
	1	36	45	19						
	2	31.8	41	27.2						
28d	3	8.0	39.2	51.8						
	4	12.8	9.0	60.1						18.1
	5		12.5	50.0	7.5					30.5
	1		67	6.0	10.0	3	8	3	3	
	2	12.7	45.7	13.6	4.5	10		4.5	4.5	
42d	3		43.3	20.0	19.3	5		3.4	10	
	4	6	6	17.5	3	4	6	18	18	21.2
	5		12.5	30.5	7.5	7	12.5			30.0
	1		58	4	10		5	4	9	10
	2	9.5	44.4	19	4.5	3.7			18.9	
56d	3		34.6	6	31	1.6			26.8	
	4		13.3		16.6		3.3	3.3	36.6	26.6
	5			30.5	7.5	7.5	12.5		12.5	30.0

Anthers cultured on the medium of Weatherhead, et al (1982) and incubated at 25°C with a 16h photoperiod. Fifty anthers were used for eadh bud stage. Results represent data from two experiments. Developmental Stages: W = white swollen anther; Y = yellow swollen anther; CBC, DBC and GN = productio of creamy-brown callus, dark brown callus and green nodular aggregates; S = shoots only produced; S & R = shoots and roots produced; C = collapsed or degenerating anthers.

could be maintained on rooting medium (MS), showing no reduction in regenerative capacity over a 3 year period, if routinely subcultured every 6 weeks (Fig 3.5).

3.3.5.2 Shoot Production Directly from the Anther Surface

Occasionally adventitious shoot arose directly from the surface of anthers (Fig 3.7a and b) cultured on the medium of Weatherhead, et al (1982). After six weeks the shoot was excised and divided then subcultured alongside anther and filament callus produced from the same anther. The anther and filament callus developed as described in 3.3.5.1. However, after two weeks in culture secondary adventitious shoot primordia formed directly from the surface of the swollen, green, shoot explants (Fig 3.8 a and b). Fresh green callus was produced from the cut end of the shoot portions. Plants were then regenerated from the parental and secondary tissues to be characterised at a later date (Chapter 4).

3.3.6 Microspore/Pollen Viability of Cultured Anthers

Data represented in Table 3.7 shows that microspore pollen viability decreases with increasing age at which anthers are inoculated. Therefore, freshly excised bud stage 1 anthers have a viability of 100%, whereas bud stage 3 anthers have a reduced viability of 88% (Fig 3.9). Furthermore, pollen viability decreases with culture age. It was found that anthers cultured from stage 1-5 buds exhibited a 56-70% decrease in viability after 28d incubation at 25°C (Table 3.7). After 28d culture at 25°C bud stage 1 and 5 anthers have a mean viability of 43% and 8.6% respectively. Moreover, after this culture period no pollen calli or pollen embryogenesis was observed in the anthers sampled.

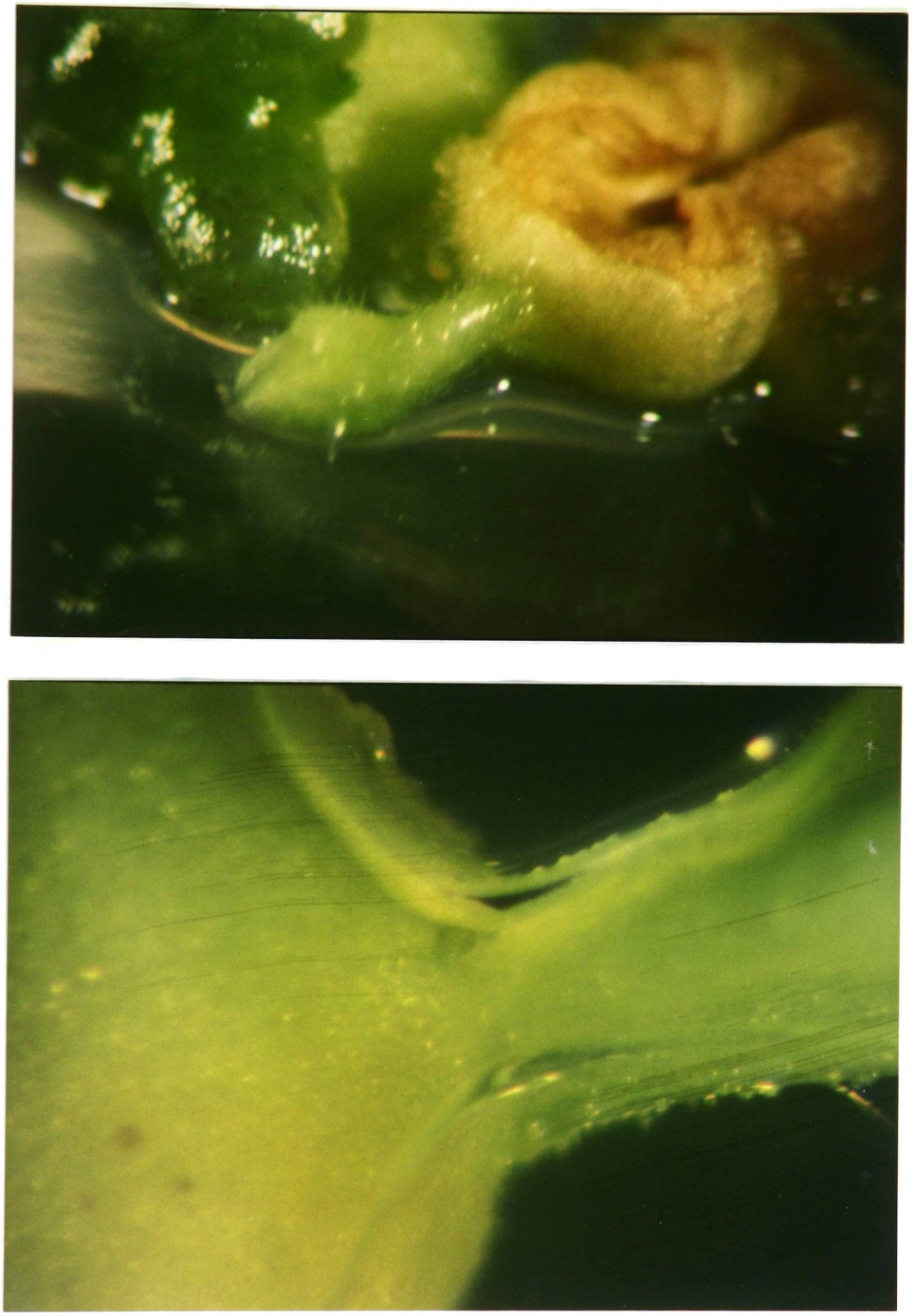


Fig 3.7 Shoot arising directly from the anther wall of an anther cultured for 35d on the medium of Weatherhead, et al (1982). a: Anterior view showing the developing shoot, creamy-brown anther callus and green filament callus (x21). b: Side view showing the point of origin of the shoot (x32).

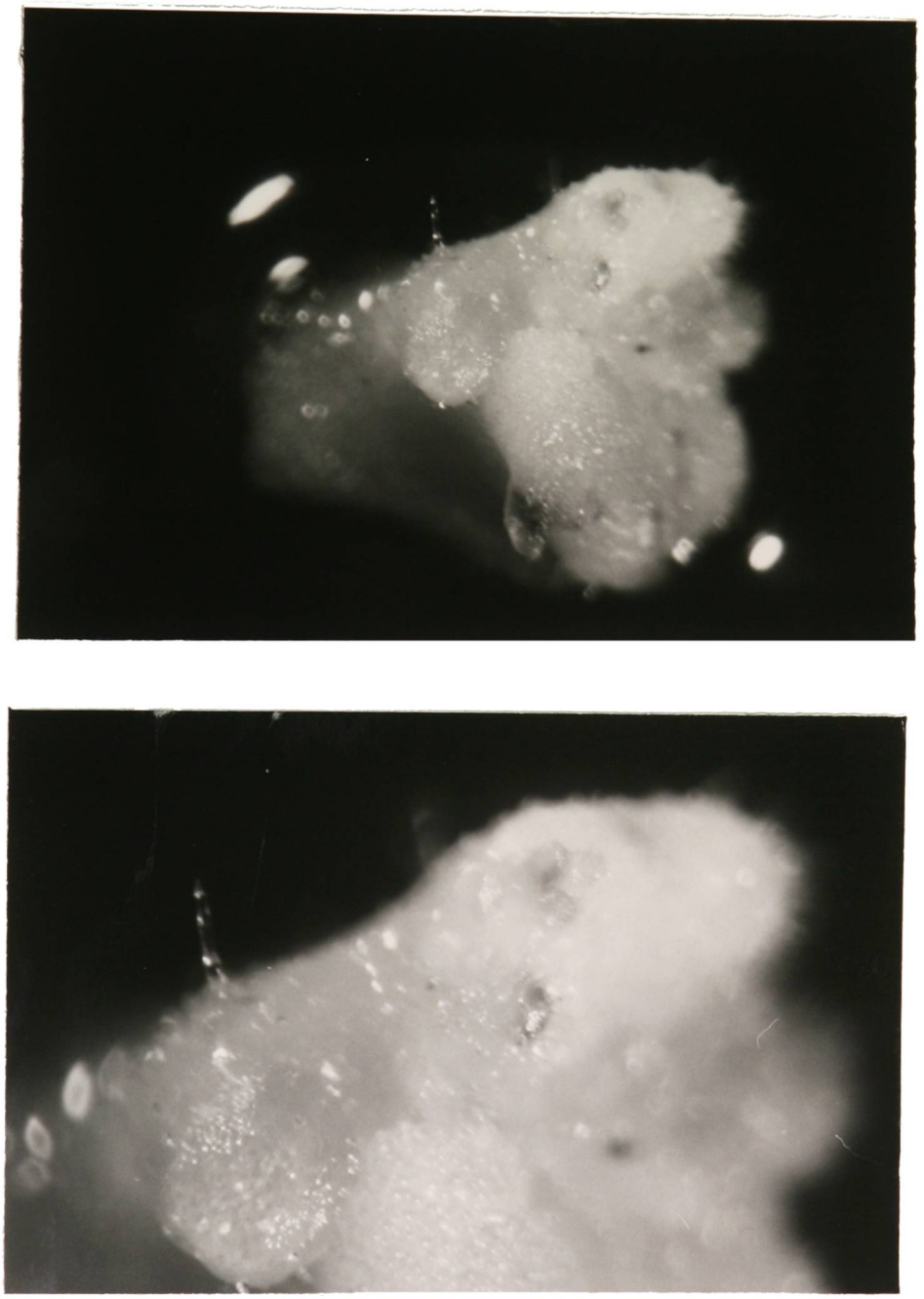


Fig 3.8 Secondary adventitious shoot primordia (SP) forming on a 14d old subcultured shoot (cultured on the medium of Weatherhead, et al (1982) that arose directly from the anther wall. a: x 75. b: x 150.

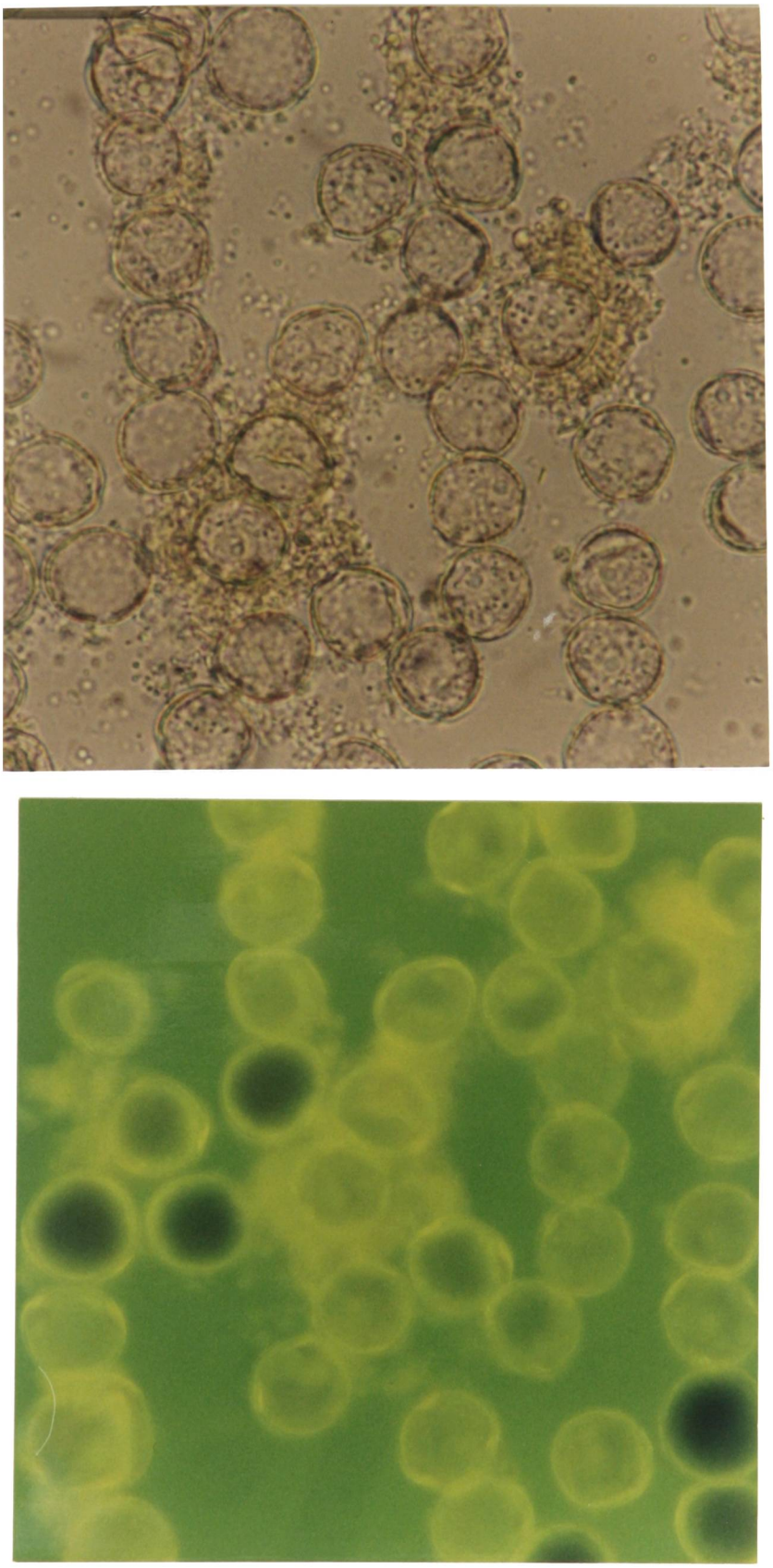


Fig 3.9 Isolated pollen from a stage 3 anther prior to culture on the medium of Weatherhead, et al (1982) (x750). a: Bright-field illumination. b: Fluorescence image of the same field, showing viable (yellow-green) and non-viable (dark green) pollen grains.

Table 3.7 Viability of Saintpaulia Microspores/pollen During Anther Culture

Culture Period (d)	Frequency of Viable Microspores/Pollen (%) Bud Stage 1	2	3	4	5	Frequency of Pollen Callus/Embryoid Formation
0	100	98.2±0.9	88.7±3.3	79.6±3.2	79.0±5.0	0
7	97.2±1.8	71.4±4.8	68.2±4.7	36.4±6.7	36.0±2.7	0
14	62.6±7.0	37.8±4.0	37.6±4.0	19.5±3.9	21.2±1.8	0
28	43.0±6.0	27.0±5.9	19.6±4.7	13.2±3.3	8.6±2.4	0

Anthers from all bud stages were cultured on the medium of Weatherhead, et al (1982) for 28d. Cultures incubated at 25°C with a 16h light period followed by 8h of darkness. At each sampling occasion, one anther was selected from each batch of 20 cultures and used to assess pollen viability, pollen callus and embryoid formation. Anthers were crushed in 10^{-6}M fluorescein diacetate in 0.4M sucrose. One hundred grains were scored per anther. Standard errors are indicated.

3.3.7 Histology and Scanning Electron Microscopy of Cultured Anthers

Sections of anthers excised from bud stages 2, 3 and 4 showed the typical anatomy of an angiosperm pollen sac (Figs 3.10 and 3.11). The anther wall consisted of an epidermis, and undeveloped endothecium, a middle layer of cells and the tapetum. The microspores/pollen grains occupied the loculus enveloped by the tapetal tissue. Directly behind the tapetum and extending inward to the vascular bundle lies parenchymatous connective tissue.

Histological and scanning electron microscopy revealed that in 13-30% of cultured anthers, callus was derived from the anther wall and connective/ pollen tissues. Furthermore, the highest frequency of such callusing was observed in stage 2 (tetrad) and stage 3 (uninucleate) anthers. (Table 3.8, Fig 3.12). However in the majority of explants (70-87%) callus originated only from anther wall tissues (Table 3.8, Fig 3.13, 3.14 and 3.15). After 21d culture on the medium of Weatherhead, et al (1982) the epidermal and middle layer of cells of the anther wall and the connective cells had increased in size (Table 3.9, Figs 3.11, 3.13 and 3.15). Whereas the connective cells showed only a 1.7 times increase in size the middle layer and epidermal cells of the anther-wall exhibited a 2 and 5 fold increase in area (Table 3.9). Furthermore, middle layer cells had undergone divisions, which were mainly periclinal giving rise to the formation of a radial row of anther wall callus cells (Fig 3.13c). Frequently meristematic growth centres, within the callus tissue, which did not differentiate further but produced parenchymatous cells from their periphery were observed (Fig 3.13c). Eventually a mass of callus tissue could be seen emerging through the ruptured anther wall epidermis (Fig 3.13a, b).

Table 3.8 Origin of Anther Callus

Bud Stage	Anther Wall	Anther Wall and Connective/Pollen
2	70.0±5.8	30.0±5.5
3	76.7±8.2	23.3±8.8
4	86.7±3.3	13.3±3.3

Anthers cultured on the medium of Weatherhead et al (1982) at 25°C under a 16h photoperiod. Callusing anthers were selected after 21-28d of culture. Origin of callus determined either by histological means or by scanning-electron microscopy of cut anthers (cf Fig. 3.10). For each bud stage 10 callusing anthers were analysed. Experiment triplicated. Standard errors are indicated.

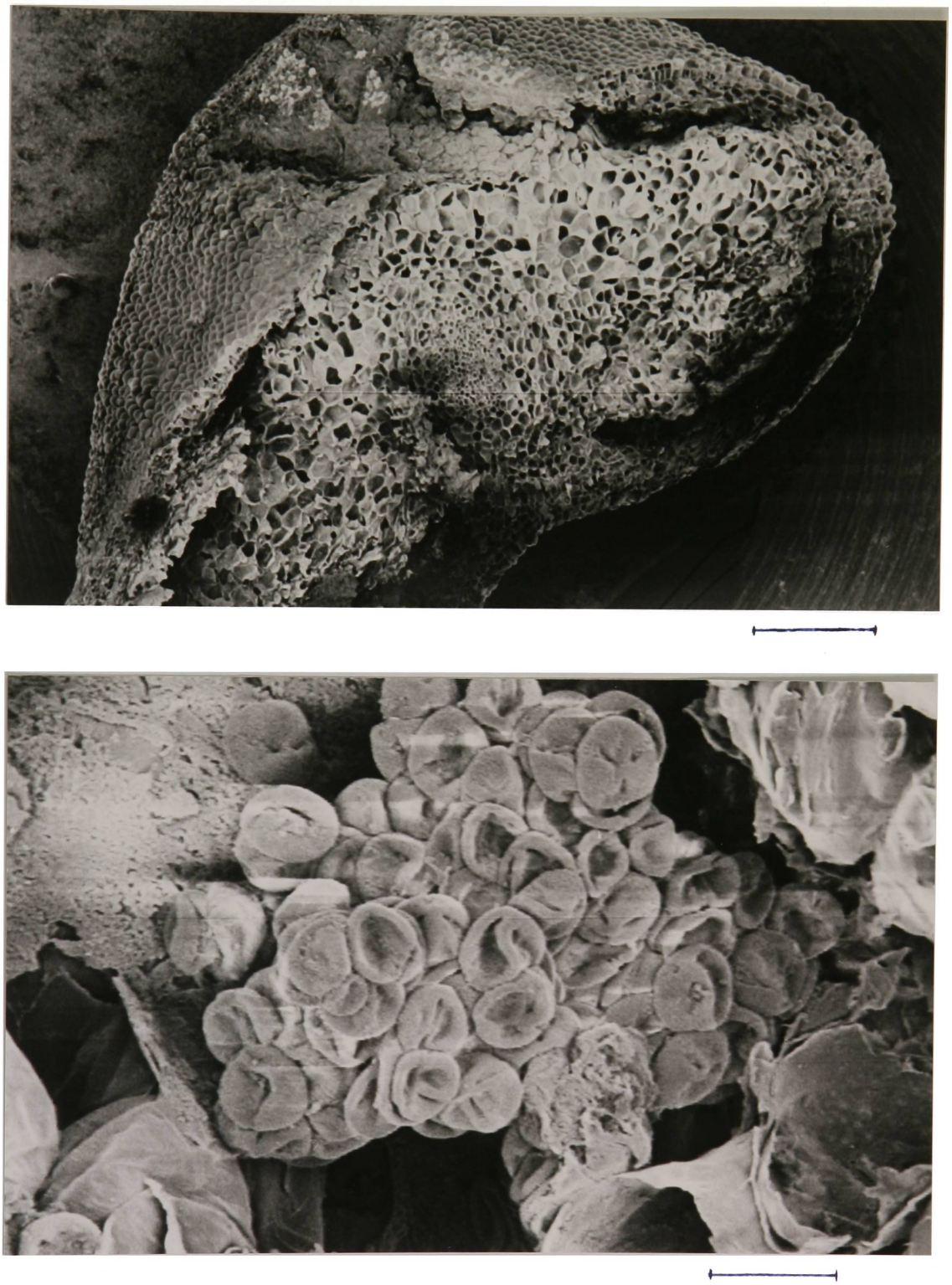
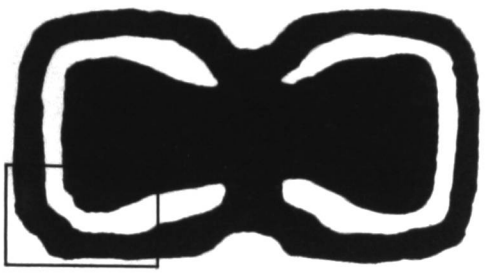


Fig. 3.10 Scanning elctron micrograph of a stage 3 anther. a: Transverse section of the anther showing anther wall (AW), loculus (L) and connective tissue (CT). Bar = 400µm. b: Pollen grains fromthe same anther section. Bar = 20µm.



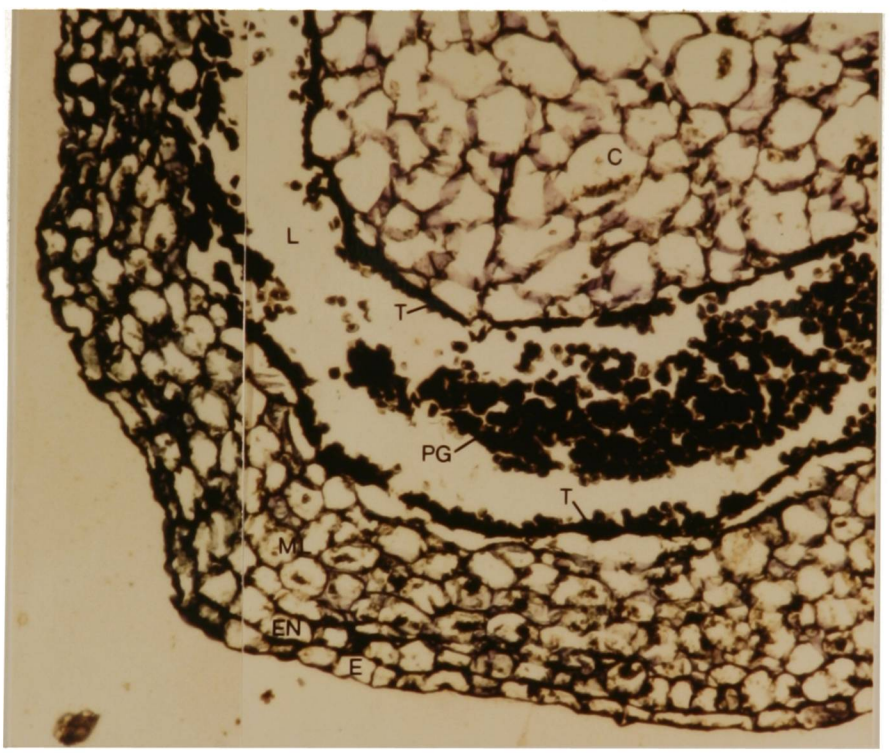


FIG. 3·11 Part of transverse section of a stage 3 anther (x428) (refer to schematic diagram). CL:cuticular layer;E:epidermis; EN:endothecium;ML:middle layer; T:tapetum;L:loculus; PG : pollen grains;C:connective.

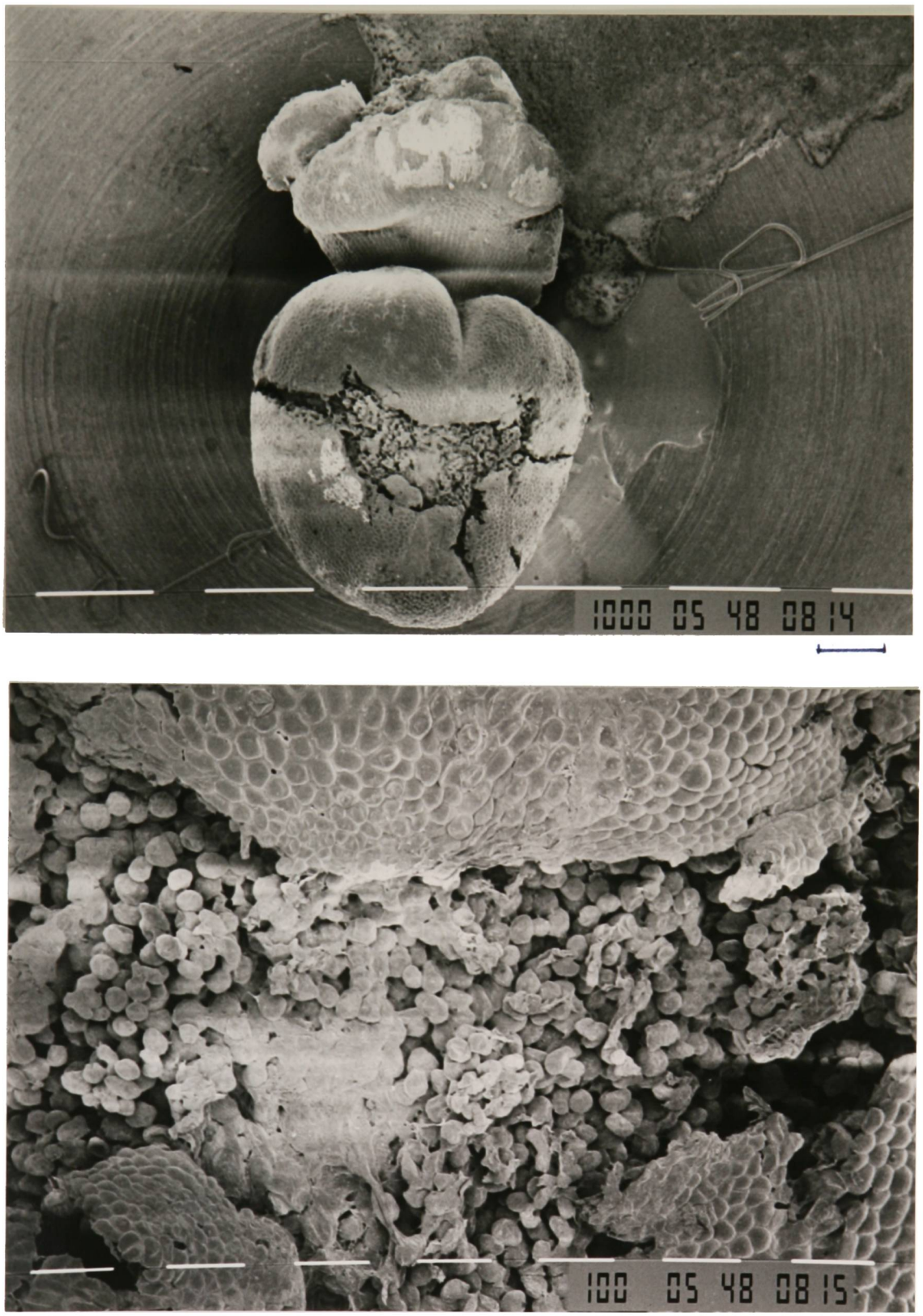


Fig. 3.12 Scanning-eclectron micrographs of a stage 3 anther cultured on the medium of Weatherhead, et al (1982) for 14d. a: Proliferating connective/pollen callus cells emerging through dehishence zone and ruptured anther walls. (bar - 1000μm). b: Enlargement of dehishence zone to show callus cells (bar = 100μm).

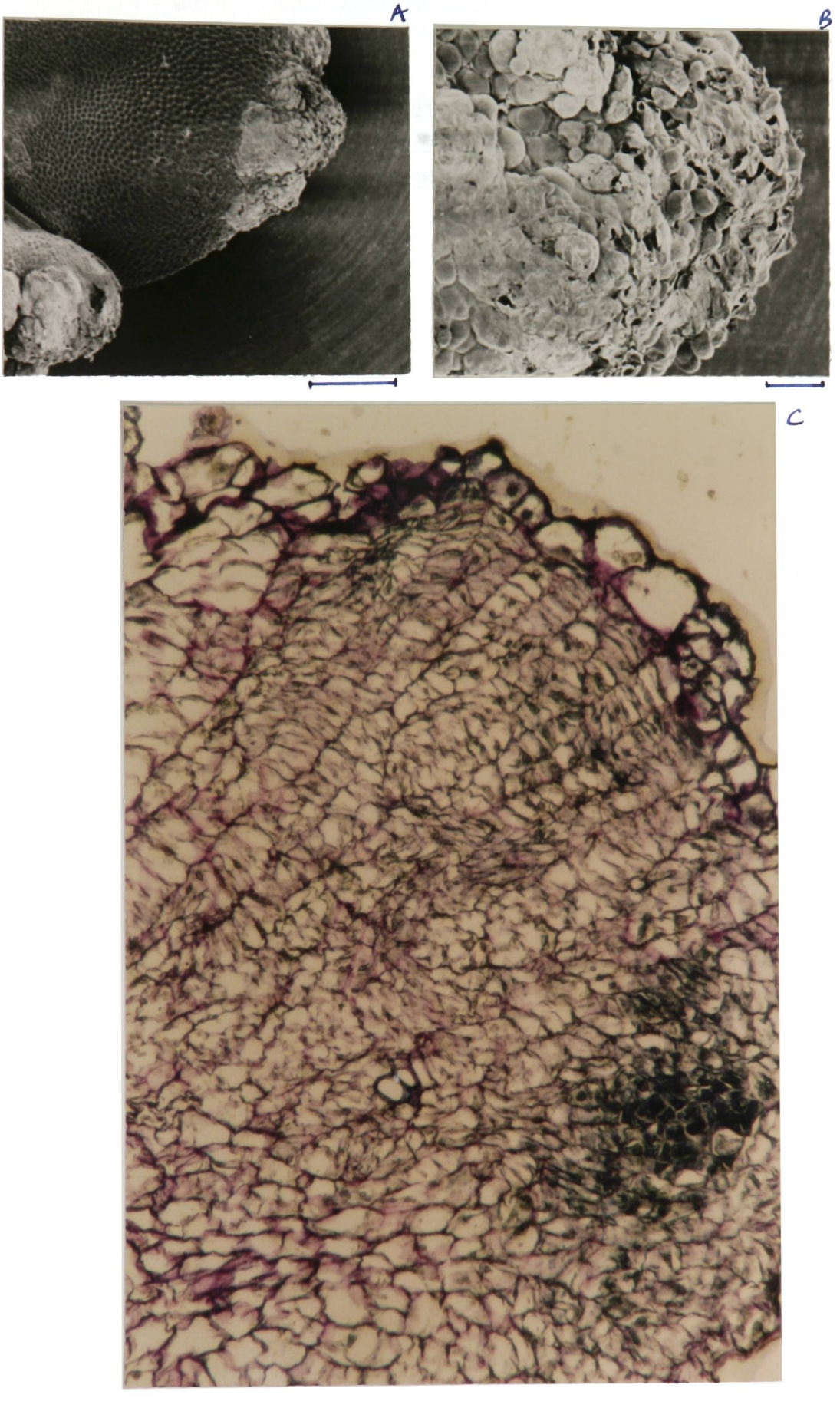

A
B
C

Table 3.9 Influence of the Medium of Weatherhead, et al (1982) on the Growth of Anther Cells

	Cell Area (um^2)	
	Day 0	Day 28
Anther Wall Epidermis	103.6 ± 6.5	539.6 ± 33.3
Anther Wall Middle Layer of Cells	133.5 ± 4.9	316 ± 13.6
Connective Cells	337.3 ± 13.4	583.1 ± 33.7
Callus Cells	-	44.7 ± 1.6
Callus Meristemic Cells	-	21.3 ± 1.0

Cell areas meausred from light micrographs of transverse sections of stage 3 control anthers (Day 0) and anthers cultured on the medium of Weatherhead, et al (1982) at 25°C (16h photoperiod for 28d. Ten anthers were used in each treatment. Standard errors are indicated.

Thereafter the anther wall callus cells rapidly proliferated (Figs 3.14 and 3.15) and by 28d had started to organise into nodular aggregates of cells (Figs 3.16) that subsequently differentiated into shoot or root primordia (Fig 3.16).

3.3.8 Influence of the Medium of Weatherhed, et al (1982) on Callus Induction from Isolated Anther Wall, Connective/Pollen and Filament Tissues

Results in Table 3.10 show that isolated anther wall, connective/pollen and filament tissues cultured on the medium of Weatherhead, et al (1982) produced callus (Fig 3.17). Anther wall and connective/pollen tissues from stage 3 and 4 anthers produced significantly more callus when compared to stage 2 anther isolates. Callus production from stage 3 and 4 anther wall tissue was 3 times greater compared to callus produced by their respective connective/pollen tissues. A maximum of 95% of stage 4 isolated anther walls produced callus. Only 2-25% of isolated filaments produced callus after 28d culture at 25°C.

3.3.9 The Effect of Anther Donor Growth Temperature on Subsequent Development of Cultured African Violet Anthers

It was found that the temperature at which flowering plants were maintained prior to the excision of anthers influenced the subsequent growth of anthers in-vitro (Table 3.11). Anthers excised from flowering plants maintained at 25°C exhibited a high frequency of anther callus production (41%) after 28d of culture. Plants grown at 20°C showed much lower frequencies of callus formation. Furthermore, the callusproduced showed a much lower morphogenetic potential. Growth of plants at 15°C resulted in a high frequency of collapsed or degenerating anthers, or anthers producing

Table 3.10 Influence of the Medium of Weatherhead, et al on Callus Induction from Isolated Anther Wall Connective/Pollen and Filament Tissues

Bud Stage	Frequency of Callus Induction (%)		
	Anther Wall	Connective/Pollen	Filament
2	55 ± 6.4	27.5 ± 4.8	2.5 ± 2.5
3	90 ± 4	32.2 ± 6.4	7.5 ± 2.5
4	95 ± 2.8	23.5 ± 4.8	25 ± 6.4

Anther wall, connective/pollen and filament tissues were isolated and cultured on the medium of Weatherhead, et al (1982) at 25°C under a 16h photoperiod (Fig 3.17). After 28d, 10 cultures per bud stage were examined for callusing. Experiment was repeated four times. Standard errors are indicated.

Fig. 3.14 Scanning electron micrograph of anther with filament attached cultured on the medium of Weatherhead, et al (1982) for 28d. a: Proliferating callus tissue (bar = 1000μm). b: Enlargement to show detail of callus cells (bar = 100μm). CL: Cuticular layer; E: epidermis; CT: Callus Tissue.

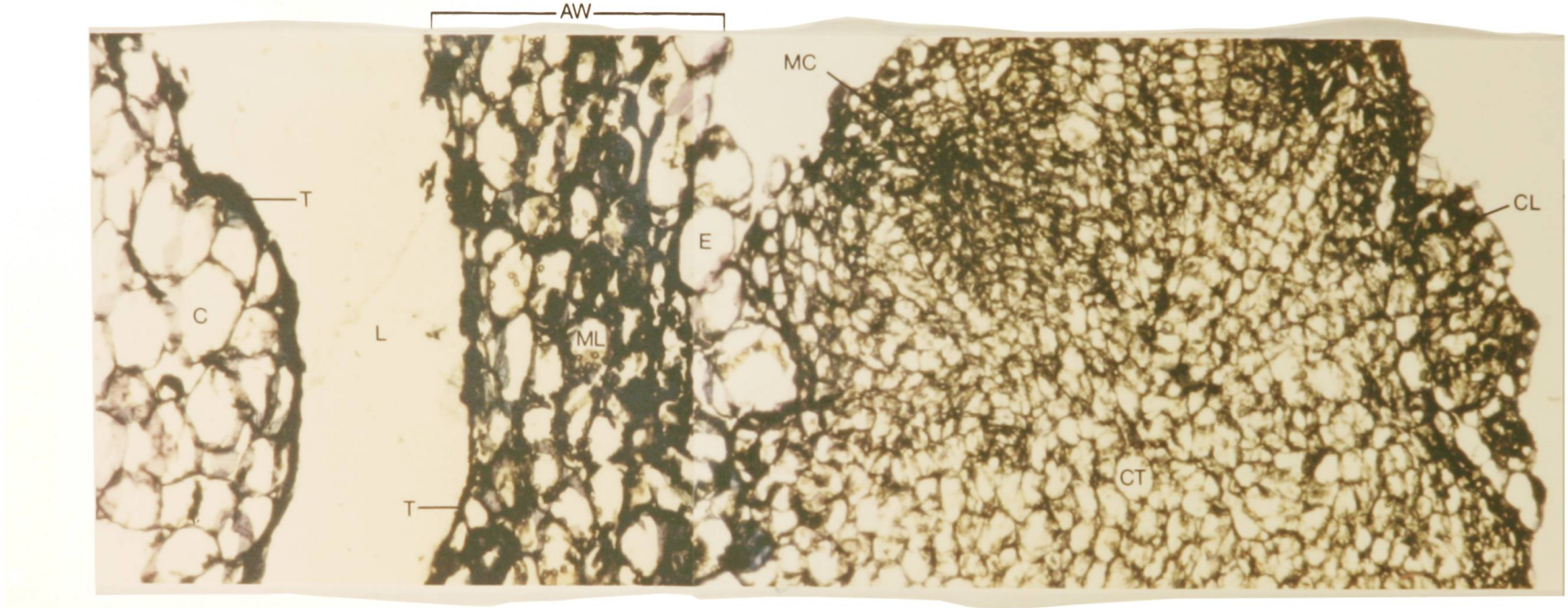


FIG. 3·15 Part of transverse section of a callusing anther cultured for 28d on the medium of Weatherhead et al. (1982) (x428). CL: cuticular layer; E: epidermis; CT: callus tissue; MC: meristematic cells; AW: anther wall; ML: middle layer; T: tapetum; L: loculus; C: connective.

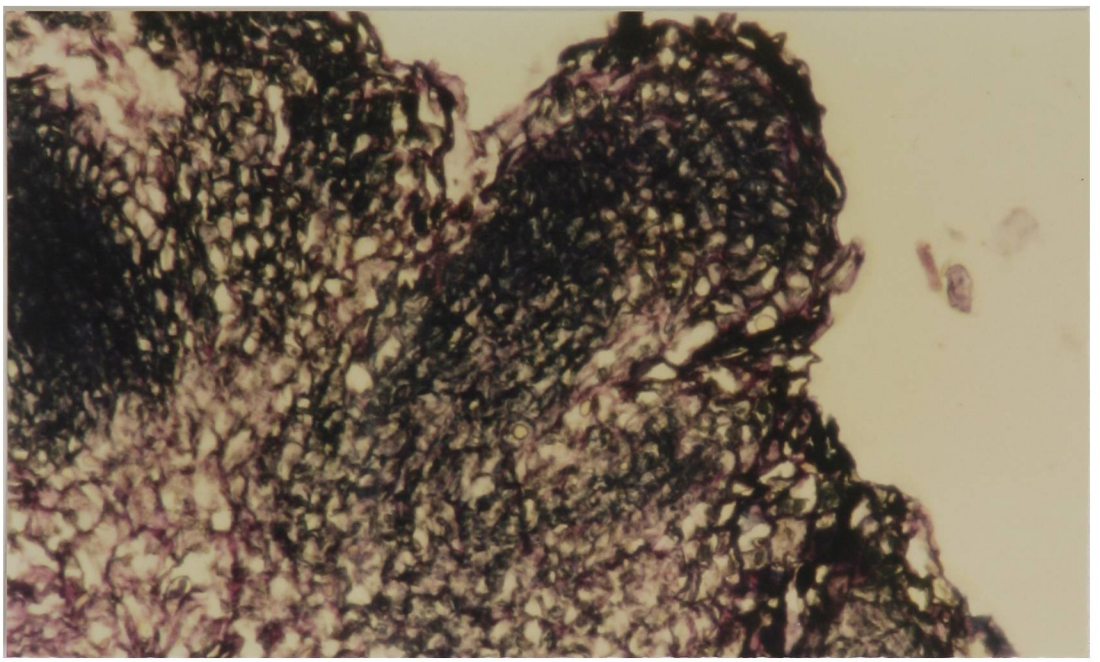


Fig. 3.16 Transerve section of an anther cultured on the medium of Weatherhead, et al (1982) for 42d. Light micrograph shows early stage of primordial nodule development on anther wall callus (x500).

Table 3.11 The Effect of the Anterh Donor Growth Temperature on Subsequent Development of Cultured African Violet Anthers

Stage of Anther Development (%)	Donor Plant Growing Temperature					
	Day 28			Day 56		
	15°C	20°C	25°C	15°C	20°C	25°C
White Swollen Anther	-	-	17.7±3.4	-	-	1.9±5.1
Yellow Swollen Anther	4.7±9.2	53.7±7.5	29.3±1.8	-	52.6±7.9	30.0±10.0
Creamy-Brown Callus	8.8±2.6	19.9±3.2	41.6±5.4	-	8.7±1.7	11.9±2.5
Dark-Brown Callus	13.3±5.8	24.7±3.2	1.5±0.3	33.3±6.1	26.3±3.5	13.9±3.6
Green Nodular Callus	-	1.7±2.5	-	-	1.7±2.0	2.5±1.0
Shoot Primordia	-	-	-	-	3.5±1.6	4.1±0.7
Root Primorida	-	-	-	-	-	1.5±0.5
Shoot and Root Primordia	-	-	-	-	3.5±2.1	20.7±6.2
Collapsed Anther	30.6±6.0	-	9.6±3.5	66.7±3.2	3.5±2.1	13.3±2.9

Anther donor plants were grown at 15°, 20° and 25°C. Photoperiod was 16h light followed by 8h darkness. Anthers were excised and cultured on medium of Weatherhead, et al (1982). Cultures were incubated at 25 C with a 16h daylength. A minimum of 50 anthers were used for each treatment. All treatments were duplicated. Standard errors are indicated.

dark brown callus that would not differentiate.

3.3.10 Influence of Low Temperature Pretreatments on In-Vitro Culture of African Violet Anthers

The results represented in Table 3.12 show that the pretreatment of flower buds at 8°C for 7 or 14d, prior to anther culture, decreased the frequency of callus formation by 14-43% when compared to control anthers excised from freshly harvested buds.

Results in Table 3.13 show that when the incubation temperature was lowered from 25°C to 15°C, the frequency of callus induction from anthers increased by 25% after 28d of culture. Further reduction of incubation temperature to 10°C was detrimental to callus formation (Table 3.13). Anthers incubated under an alternate 15°C (16h light) and 25°C (8h darkness) growth regime induced significantly more callus when compared to anthers grown at 25°C. However, comparing the callus induction data of Table 3.13 to the growth analysis results (Table 3.14) shows that although callus formation is increased by culturing anthers at 15°C or 15°C (16h light)/25°C (8 darkness) this benefit is far outweighed by the greater number of shoots produced at the higher temperature (25°C) after the same period of incubation time.

3.3.11 Culture of Anthers in Darkness

It was found that culturing anthers in the dark did not increase the frequency of callus formation. The data in Table 3.15 showed no significant difference in callus formation in anthers cultured in the light or dark. In both light and dark treatments callus induction increased with increasing anther age.

Table 3.12 Influence of Low Temperature Bud Pretreatments on Callus Induction in Cultured Anthers

Time of Cold Pretreatment(d)	Induction Frequency of Callus (%)
0	48.0±3.0
7	34.4±4.4
14	4.7±0.8

Buds were harvested and pretreated at 8°C for 7 or 14d. Anthers were excised and cultured on the medium of Weatherhead, et al (1982). Cultures were harvested after 28d of incubation at 25°C with a 16h daylength. Fifty replicates were used for each treatment. Experiment was duplicated. Standard errors are indicated.

Table 3.13 Influence of Incubation Temperature on Callus Induction of Anthers

Culture Time (days)	Induction Frequency of Callus (%) Incubation Temperature (°C)			
	10°	15°	15°(16h)/ 25°(8h)	25°
14	54.5±1.5	95.7±4.4	80.9±4.9	71.9±2.8
28	52.1±2.1	91.7±8.4	81.9±2.7	66.8±11.4
35	43.0±3.0	91.7±8.4	74.0±10	58.7±3.6

Bud stage 2, 3 and 4 anthers were cultured on the medium of Weatherhead, et al (1982) at various incubation temperatures. Photoperiod was 16h light followed by 8h of darkness. Explants were harvested after 56 d. Fifty replicates were used for each treatment. Experiment was duplicated. Results were combined prior to analysis. Standard errors are indicated.

Table 3.14 Influence of Incubation Temperature on Explant Area, Shoot and Root Production After 56 Days of Culture

		Incubation Temperature (^{o}C)			
	Day 0	10^{o}	15^{o}	15^{o}(16h)/ 25^{o}(8h)	25^{o}
Callus Area (mm^2)	5.7±0.6	15.1±0.7	57.9±12.1	51.5±6.8	121.7±27.5
Shoot No	0	0.5±0.05	1.1±0.9	0.4±0.1	42.7±13.8
Root No	0	5.9±0.5	10.9±7.1	10.9±2.2	7.0±2.0

Bud stage 2, 3 and 4 anthers were cultured on the medium of Weatherhead, et al (1982) at various incubation temperatures. Photoperiod was 16h light followed by 8h of darkness. Explants were harvested after 56d. Fifty replicates were used for each treatment. Experiment was duplicated. Results were combined prior to analysis. Standard errors are indicated.

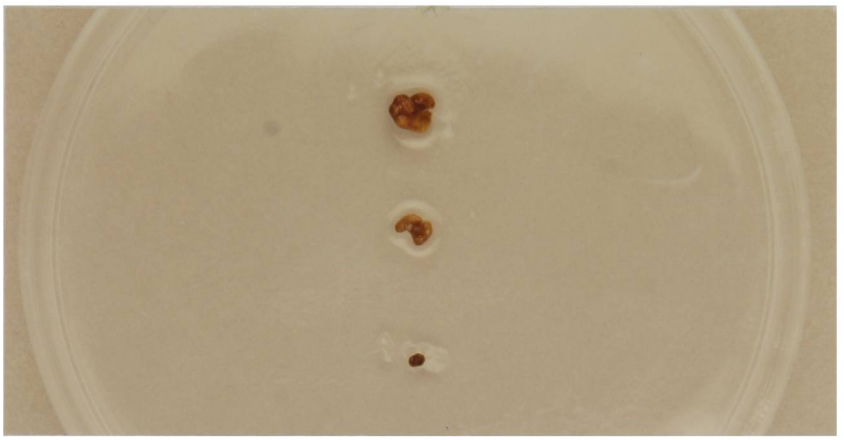
Table 3.15 Culture of Anthers in the Darkness

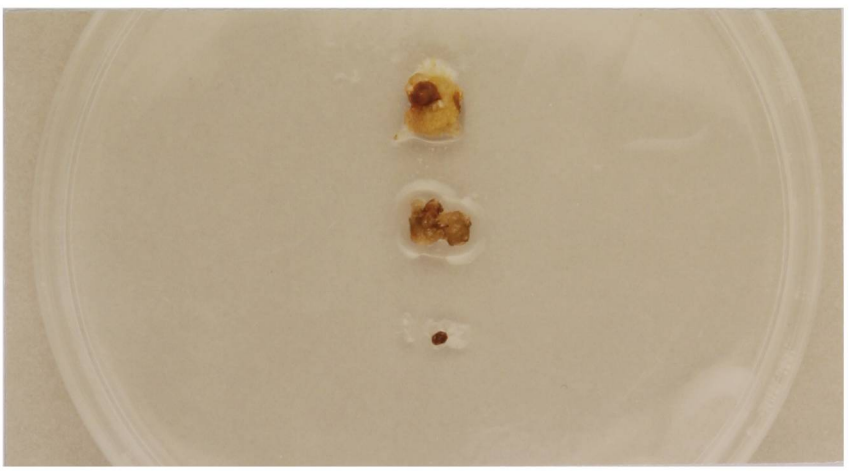
Anther Stage	Frequency of Callusing Anthers (%) Light Grown	Dark Grown
2	47.9±3.1	48.5±0.6
3	64.8±11	57.6±7.6
4	65.0±7.0	73.5±2.5

Anthers cultured on the medium of Weatherhead, et al (1982) for 28d. Cultures incubated at 25°C under a 16h light period followed by 8h of darkness. Thirty anthers were used for each treatment. Experiment was duplicated. Standard errors are indicated.

Fig. 3.17 Culture of stage 3 anther wall (top explants), connective/pollen tissues (middle explants) and filament (lower explant) on the medium of Weatherhead et al (1982). a: Day 0 (x2). b: Day 28 showing callus formation from both the anther wall and connective/pollen tissues but ot the isolated filament (x2).

Fig. 3.17 Culture of stage 3 anther wall (top explants), connective/pollen tissues (middle explants) and filament (lower explant) on the medium of Weatherhead et al (1982). a: Day 0 (x2). b: Day 28 showing callus formation from both the anther wall and connective/pollen tissues but ot the isolated filament (x2).





3.3.12 Effect of Sucrose

The effect of sucrose concentration, in the medium of Weatherhead, et al (1982), on the callus induction from cultured anthers was determined. The results of the experiments are shown in Table 3.16. It was found that the frequency of callus formation is highest in 3% sucrose; moreover, the induction frequency decreased with the increase of sucrose concentration in the medium. If the anthers are cultured with sucrose over 6% the frequency of callus formation decreased, indicating that a concentration of sucrose higher than 6% is deleterious to the induction of callus. A maximum callus induction frequency of 61% was obtained by stage 4 anthers cultured on medium containing 3% of sucrose.

3.3.13 Effect of Auxin and Cytokinin

The influence of auxin and cytokinin concentration on the induction of callus (Table 3.17), and the subseuqnet development of shoots and roots from cultured anthers was investigated (Table 3.18). The MS medium, modified in hormone concentrations by Weatherhead, et al (1982) was subject to further modification with respect to concentration and combination of hormones. Three hormone treatments were investigated and it was found that callus induction and subsequently shoot and root differentiation increased with increasing anther age (Tables 3.17 and 3.18). Furthermore, anthers cultured on the medium of Weatherhead, et al (1982) supplemented with NAA dn BAP (1:0.5mgl^{-1}) always exhibited a higher frequency of callus induction and differentiation, than that of the other two hormone treatments tested. The greatest induction rates were obtained from stage 4 anthers incubated the above medium.

Table 3.16 Influence of Sucrose Concentration on Callus Induction of Cultured Anthers

Anther Stage	Frequency of Callusing Anthers (%) Sucrose Concentration (%)					
	0%	3%	6%	9%	12%	15%
1	0	4.5±4.5	0	0	0	0
2	0	33.5±7.5	20.4±6.8	16.3±2.7	3.2±3.1	7.3±2.7
3	0	57.5±2.0	35.9±5.9	19.2±7.7	11.2±7.7	6.7±6.7
4	0	61.0±9.3	40.7±9.5	24.4±2.9	10.8±3.4	3.6±3.6
5	0	35,4±3.0	23.5±7.2	0	0	0

Anthers cultures for 28d on the medium of Weatherhead, et al (1982). Cultures incubated for 28d at 25°C with a photoperiod of 16h light followed by 8h of darkness. A minimum of fifty anthers was used for each treatment. All treatments were duplicated. Standard errors are indicated. Plants were regenerated from the differentiated callus tissues to be characterised at a later date (Chapter 4).

Table 3.17 Effect of Various Concentrations of NAA and BAP on Callus Formation from Anthers

Bud Stage	Induction Frequency of Callusing Anthers (%) Concentration of NAA:BAP mgl^{-1}		
	1:0.5	1:1	0.5:1
2	36 ± 2.0	23 ± 2.0	16.0 ± 1.0
3	43 ± 4.0	33 ± 4.0	32 ± 3.0
4	62 ± 5.0	49 ± 2.0	37 ± 4.0

Frequency of callus induction from cultured anthers. Anthers from stage 2, 3 and 4 buds were cultured for 28d on the medium of Weatherhead, et al (1982) supplemented with NAA and BAP at various concentrations. Cultures were incubated at 25°C under a 16h light period followed by 8h darkness. One hundred anthers were used for each treatment. All treatments were duplicated. Standard errors are indicated. Plants were regenerated from the anther-derived shoots to be characterised at a later date (Chapter 4).

Table 3.18 Effect of Various Concentrations of NAA and BAP on the Frequency of Shoot and Root Induction from Cultured Anthers

	Concentration of NAA:BAP (mgl^{-1})								
	1:0.5			1:1			0.5:1		
Bud Stage	Shoot only	Root only	Shoot & Root	Shoot only	Root only	Shoot & Root	Shoot only	Root only	Shoot & Root
2	$5.0^{\pm}1$	$4.0^{\pm}3$	$26.0^{\pm}3$	$12.0^{\pm}3$	$4.0^{\pm}1$	$9.0^{\pm}3$	0	0	$16.0^{\pm}2$
3	$12.0^{\pm}3$	$6.0^{\pm}2$	$32.0^{\pm}3$	$9.0^{\pm}6$	$1.0^{\pm}1$	$23.0^{\pm}2$	$8.0^{\pm}3$	$3.0^{\pm}1$	$12.0^{\pm}2$
4	$5.0^{\pm}4$	$2.0^{\pm}2$	$41.0^{\pm}5$	$11.0^{\pm}2$	$2.0^{\pm}1$	$34.0^{\pm}5$	$9.0^{\pm}3$	$2.0^{\pm}1$	$30.0^{\pm}4$

Frequency of shoot and root differentiation from cultured anthers. Anthers from stage 2, 3 and 4 buds were cultured for 28d on the medium of Weatherhead, et al (1982) supplemented with NAA and BAP at various concentrations. Cultures were incubated at 25°C under a 16h light period followed by 8h darkness. One hundred anthers were used for each treatment. All treatments were duplicated. Standard errors are indicated. Plants were regenerated from the anther-derived shoots to be characterised at a later date (chapter 4).

3.3.14 The Effect of Ferric Ions

it was found that culturing antherson the medium of Weatherhead, et al (1982) without chelated iron was highly deleterious to callus production (Table 3.19). Over 60% of anthers either degenerated or produced slow growing dark brown callus that did not differentiate under the prescribed growth conditions. However, addition of ferric ions to the medium caused a four fold increase in anthers producing creamy brown callus that further differentiated to produce shoots and roots.

3.3.15 The effect of Activated Charcoal

Attempts to increase the callus induction frequency by culturing anthers onthe medium of Weatherhead, et al (1982) with the addition of 1% activated charcoal proved to be unsuccessful (Table 3.20). Anthers innoculated on the medium of Weatherhead, et al (1982) supplemented with hormones and containing charcoal produced a slow growing dark brown callus that did not differentiate. Whereas, control anthers produced a creamy-brown callus that differentiated into shoot and root primorida after 8 weeks of incubation.

3.3.16 The Effect of Polyvinylpolypyrrolidone (PVPP)

The influence of PVPP on callus induction from cultured anthers was negligible as shown in the results presented in Table 3.21. It can be seen from the data that when the medium of Weatherhead, et al, (1982) was supplemtned with 0.1-1.5% PVPP the callus induction frequency from cultured anthers is equal to or lower than the control value of 66%.

Table 3.19 Influence of Ferri Ions on Callus Induction from Cultured Anthers

Stage of Development	Induction Frequency	
	Without Ferric Ions	With Ferric Ions
White Swollen Anther	23.4±1.2	7.8±2.0
Yellow Swollen Anther	-	15.7±4.4
Creamy Brown Callus	14.9±2.4	61.2±7.5
Dark Brown Callus	36.2±3.8	7.4±2.9
Collapsed or Degenerating Anthers	25.5±4.1	7.8±2.9

Anthers cultured on the medium of Weatherhead, et al (1982) with or without Ferric ions. Cultures were incubated at 25°C for 28d under a 16h light period followed by 8h darkness. A minimum of 50 anthers were used for each treatment. Treatments were triplicated. Standard errors are indicated.

Table 3.20 Influence of Activated Charcoal on Callus Induction from Cultured Anthers

Bud Stage	Basal Weatherhead et als Medium	Basal Weatherhead et als Medium + 1% Activated Charcoal	Weatherhead et als Medium + 1% Activated Charcoal	Weatherhead et als Medium
2	0	0	0	20 ± 2
3	0	0	$32\pm$DBC	46 ± 6
4	0	0	$24\pm$DBC	56 ± 6
5	0	0	$50\pm$DBC	50 ± 4

Anthers excised from stage 2, 3 4 and 5 buds and cultured on the medium of Weatherhead, et al (1982) supplemented with or without hormones and activated charcola (1%). Cultures were incubated at 25°C for 28d under a 16h light period followed by 8h darkness. Fifty anthers were used for each treatment. All treatments were duplicated. Standard errors are indicated. DBC = slow growing non-morphogenic dark brown callus.

Table 3.21 Effect of PVPP on Callus Induction from Cultured Anthers

PVPP Concentration (%)	Induction Frequency	
	Hormone Free	Hormone Added
0	0	66±1.8
0.1	10±6	52±8.0
0.5	6±2	66±1.4
1.0	0	40±12
1.5	2±2	60±3.1

Anthers were cultured on the medium of Weatherhead, et al (1982) or on basal Weatherhead, et al medium. PVPP was added at concentrations between 0.1-0.5%. Cultures were incubated at 25°C for 28d under a 16h light period followed by 8h darkness. A minimum of thirty anthers was used for each treatment. All treatments were triplicated. Standard errors are indicated

Table 3.22 The Effect of Developmental Stage and Cold temperature Pretreatments on Anther Dehiscence and Liberation of African Violet Pollen into Liquid Culture Medium

Bud Stage	Dehiscence Frequency (%) Untreated Anthers	Pretreated Anthers
2	24.5±8.5	20.0±0
3	47.5±15.5	50.0±20
4	70.5±5.5	80.0±6
5	100.0±0	100.0±0

Anthers were dissected from untreated and cold-pretreated (-8°C for 7d) buds. After dissection a minimum of 50 anthers from each bud stage were floated on liquid A medium. All dishes were sealed with Parafilm and incubated at 25°C in the darkness. After 14d the number of dehisced anthers was recorded. Results show the mean dehishence frequency from three experiments. Standard errors are indicated.

3.3.17 Isolated Pollen Grain Culture

3.3.17.1 Hanging Drop Culture

Preliminary experiments to test the influence of MS media and sucrose concentrations on the division of pollen in hanging-drop cultures (Fig 3.18) proved to be unsuccessful. After 56d, divisions were not observed in pollen mechanically isolated from stage 2, 3 and 4 anthers and suspended in MS medium at concentrations of 100%, 10% and 1%. Addition of sucrose at levels of 3, 6, 10, 12 and 15% to the three concentrations of MS media and incubating the cultures either in the light or the dark did not initiate pollen division.

3.3.17.2 Anther Float Culture

The results in Table 3.23 show that cultures initated at the tetrad pollen stage 2 do not open to any great extent and only 25% of anthers shed pollen into the medium after 14d of culture. With increase in anther age, however, the frequency of anthers dehiscing increases to 100% in stage 5 anthers. Chilling of buds at 8°C for 7d has no effect on the frequency of Blue Rhapsody anther dehishence.

The above above 'non-mechanical' culture was exploited to evaluate the influence of temperature pretreatments onthe induction frequency of microspore division. Buds were pretreated at 4°, 8°, 15°, 20°, 25° and 30°C for varying lengths of time ranging from 0-28d. Anthers were then excised and floated on A medium (major salts (half strength) and Fe-EDTA(full strength) of Murashige and Skoog (1962) medium plus 2% sucrose, pH5.5) and AGSI medium (A medium plus L-glutamine 800mgl^{-1}, L-serine 100mgl^{-1} and myo-inosital 5,000mg^{-1}). After 28 and 56d of culture pollen divisions were not observed in any of the above treatments.

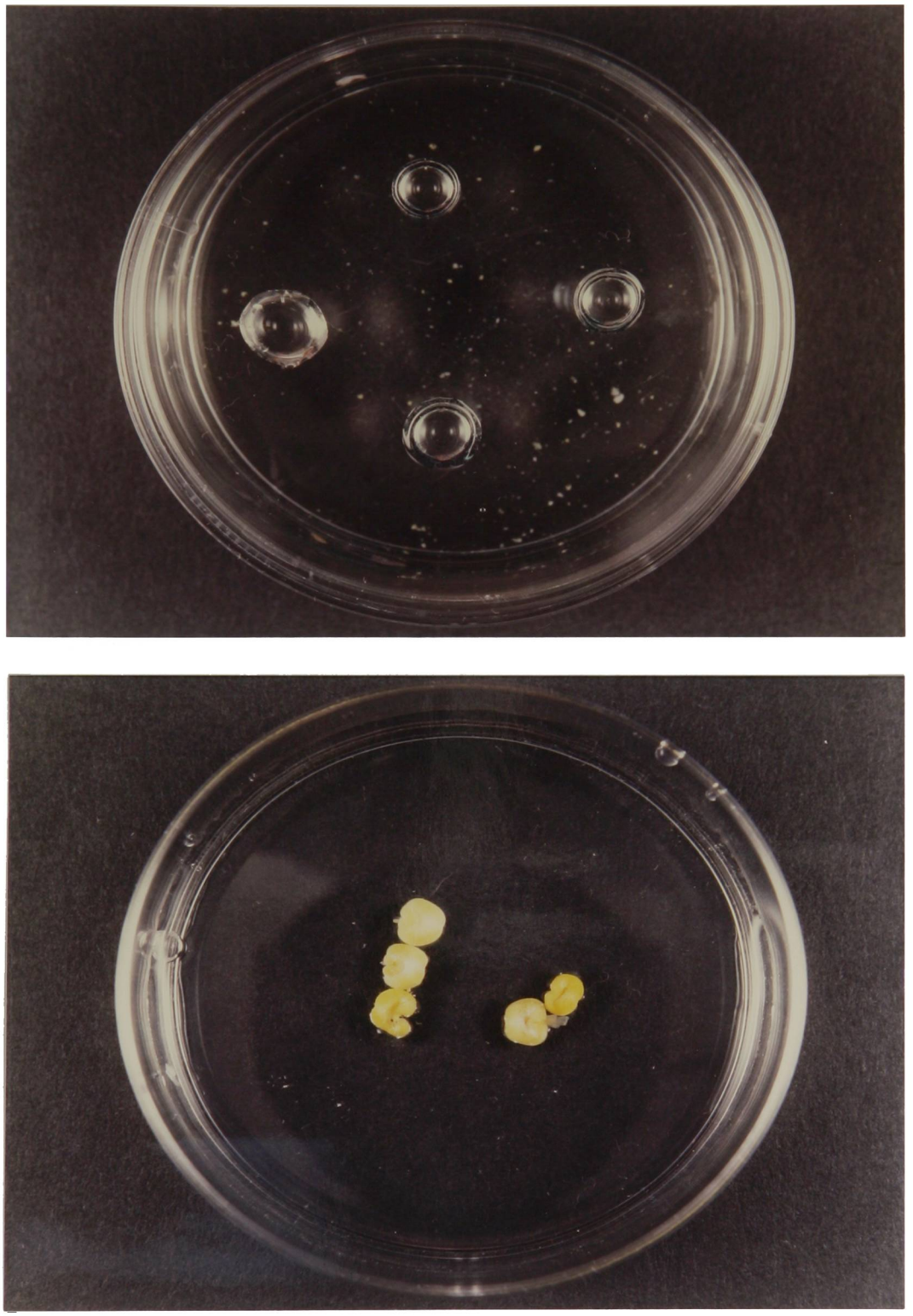


Fig. 3.18 Methods of pollen culture. a: hanging-drop culture. Pollen isolated from anther homogenates and suspended in droplets of MS medium (x19). b: Anther float culture. Anthers were dissected from untreated and cold-pretreated buds and floated on 5ml of A or AGSI medium contained in 5cm Petri dishes (x1.94).

Table 3.23 The Influence of Anther Nurse Tissue Callus on the Frequency of Multi-Cellular African Violet Pollen

Bud Stage	Frequency of Nurse Tissue Having Adjacent Multicellular Pollen (%)	Frequency of Multicellular Pollen (%)
2	0	0
3	$4.5^{\pm}1.5$	$7.3^{\pm}1.4$
4	$1.5^{\pm}0.5$	$4.0^{\pm}1.0$
Control	0	0

Anthers were cultured on the medium of Weatherhead, et al (1982). A sterile filter paper disc was placed over each anther and 10^3-10^4 pollen grains, from the same pollen stage were pipetted onto the disc. Controls were prepared in an identical manner, however the pollen grains were pipetted onto filter paper discs placed directly on the agar solidified medium. All cultures were incubated at 25^{o}C with a photoperiod of 16h light followed by 8h darkness. Hundred anthers were plated for each bud stage. Experiment was duplicated. Standard errors are indicated.

3.3.17.3 Pollen Culture on Anther Nurse Tissue

After six weeks inculture none of the mechanically isolated pollen grains pipetted onto the filter paper overlying the callusing anther nurse tissue were observed to be dividing. However, on the surface of the solidified medium adjacent to stage 3 and 4 nurse tissue explants there were rows, of what appeared to be,dividing pollen (Fig 3.19). When isolated and examined, using bright field and fluorescence microscopy, it was found that they were multicellular pollen grains. Figure 3.20 shows one such grain which has undergone successive divisions of the nuclei causing the eventual rupturing of the pollen grain wall. The origin of the multicellular pollen could either be from the pollen initially innoculated on the filter paper discs or from the dehishence and sheding of pollen from the anther, nurse tissue. Data in Table 3.26 indicates that multicellular pollen was derived only from bud stage 3 and 4 anther-nurse tissue explants. Furthermore, pollen adjacent to stage 3 explants produced the highest number of microspores that developed into multicellular pollen grains. No further division or development was observed in the remaining multicellular pollen grains.

3.4 Discussion

Microsporogenesis in African violet Var Blue Rhapsody follows the typical developmental pattern described for Angiosperm pollen (Sunderland and Dunwell, 1977). For descriptive purposes, the developmental sequence can be divided into three distinctive phases, namely, the formation of spore, tetrads from each pollen mothercell, the development of individual microspores after release from the mother cell wall, and the maturation of microspores into pollen grains. The third phase begins with

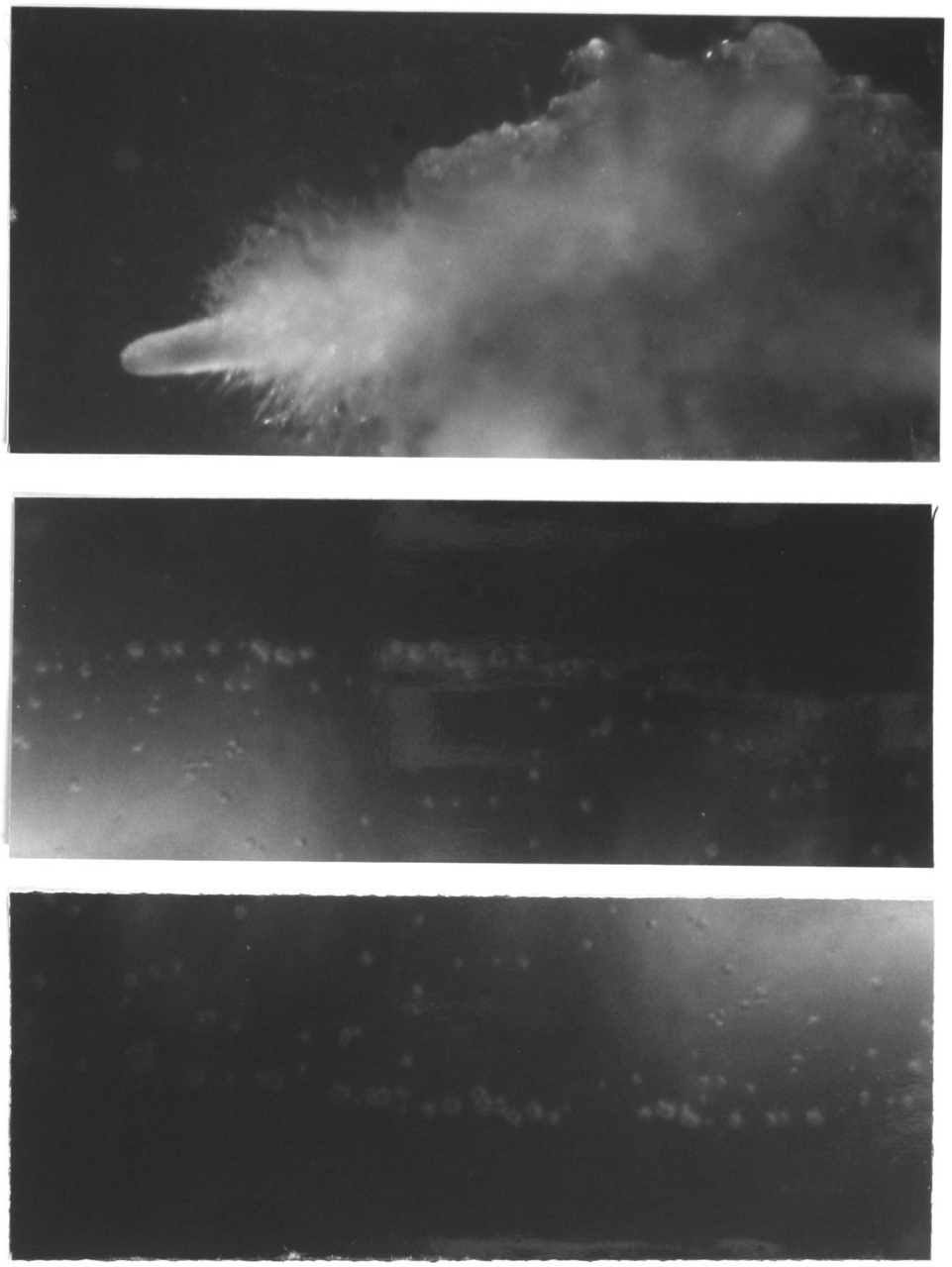


Fig. 3.19 Photomicrographs showing a row of stage 3 normal and multicellular pollen on the solidified media surface adjacent to a root growing from an anther-nurse tissue callus. Anthers incubated on the medium of Weatherhead, et al (1982) at 25°C for 42d. a: Row of normal and multicellular pollen (arrowed) neighbouring anther nurse tissue (x23). b: Enlargement to show row of pollen (x90). c: Close up to show heart-shaped multicellular pollen (x180). (cf Fig. 3.20b).

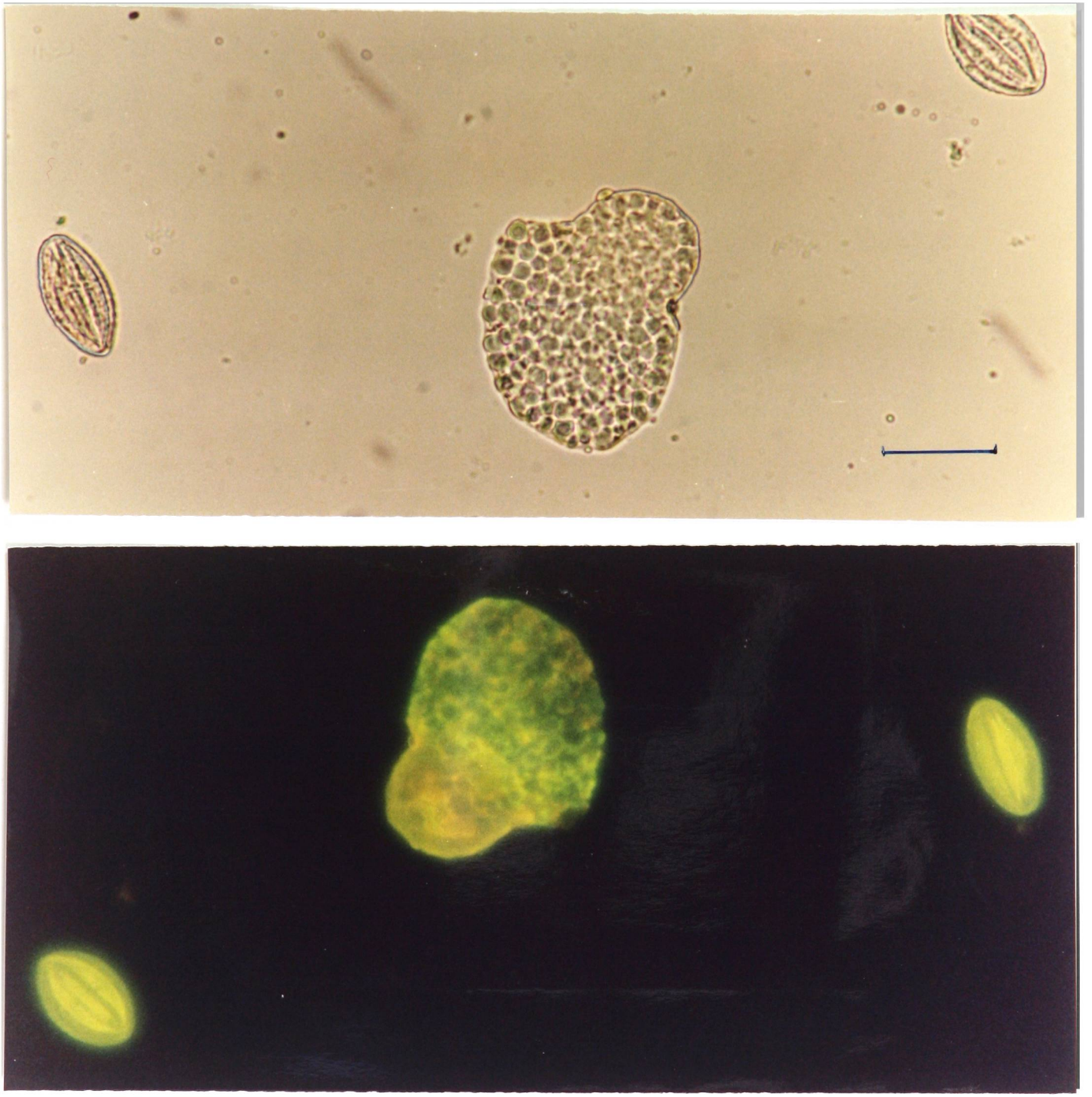


Fig. 3.20 Stage 3 normal (left and top right) and multicellular pollen (middle) isolated from the media surface adjacent to an anther-nurse tissue callus after 42d culture on the medium of Weatherhead, et al (1982). a: Bright-field illumination showing cell mass. b: Fluorescence image of the same field showing viable (yellow-green) multicellular and normal pollen grans. Bar = 20μm.

the first pollen division which leads to the formation of a small generative and a much larger vegetative nucleus.

Previous workers have shown that the degree of synchrony between the developing spores in an anther varies with the species (Sunderland and Wicks, 1971). In this investigation all microspores extracted from an anther usually exhibited the same stage of development, that is pollen development in an anther is relatively synchronous. However, anthers collected from buds 8.6±0.5mm in diameter are exceptions. Here, mitotic and mature starchy binucleate pollen grains were observed in the sampled populations. This indicates that a relatively large part of the spore population lags behind the rest, and a dimorphic pollen population is produced. Furthermore, the two populations were readily distinguished. The normal, starchy grains stained deeper with acetocarmine and were significantly larger in diameter compared to the weaker staining 'atypical' pollen. The occurence of dimorphic pollen, that is the presence in an anther of pollen of two different morphologies and staining characteristics (section 3.1), has been reported by other workers in a variety of species (Clapham, 1971; Wenzel and Thomas, 1974; Dale, 1975; Sunderland and Dunwell, 1977; Horner and Street, 1978; Zhou, 1980; Heberle-Bors, 1982). As of yet no such dimorphism has been reported in Saintpaulia.

Past interpretations of pollen dimorphism have indicated that the smaller atypical pollen grains with a cytoplasm that stains less intensely with acetocarmine is embryogenic polle which gives rise to pollen embryoids or callus. (Horner and Street, 1978; Zhou, 1980; Ta. and Hallorans, 1982; Rashid, 1983). Indeed, evidence collected over the last fifteen years, from other studies of pollen

dimorphism is now so comprehensive that the phenomenon is much more widespread than initially assumed. Therefore, it is highly probably that the atypical grains observed in this study are potentially embryonic pollen.

Previous work on Saintpaulia has indicated that bud diameter is not a good indication of anther stage since anthers from the same bud may be in different stages of development (Hughes, et al, 1975). However, no detailed analysis was reported to substantiate this suggestion. In this study it was observed that as pollen development in a Blue Rhapsody anther was relatively synchronous (excluding anthers from buds 8.6 ± 0.5mm in diameter, see above) it was possible to correlate the stage of pollen development with bud diameter. Five stages were defined according to the stage of the majority of pollen in an anther. The stages described are an adaption of the six anther stages defined by Sunderland (1974). They served as a useful guideline to identify the critical period (late uninucleate pollen, Weatherhead, et al, 1982), for Blue Rhapsody anther culture, thus saving time, in many instances, of staging anthers by cytological criteria. Therefore to obtain Blue Rhapsody anthers within the critical period defined by Weatherhead, et al (1982) anthers from bud stage three should be selected.

Hughes, et al (1975) and Smith, et al (1981) described low levels of plantlet production typically 1-5 plantlets per anther from 3-18% of anthers cultured on Blaydes's medium. Comparable results were obtained in this study. However, the above workers reported that plantlets could be seen growing directly from the anther surface or from light tan callus. In this investigation shoots were only observed differentiating from callus masses. Data in this study

confirms that of Weatherhead, et al (1982). The most effective medium,with respect to callus induction, shoot and root production from African violet anthers, is MS supplemented with NAA:BAP, 1:0.5mgl^{-1}. Furthermore, using the same commercial line described by the above workers, the maximum frequency of callusing anthers is 73%, more than twice that recorded previously. After eight weeks in culture shoot production from a cultured anther ranged from a mean of 6-29 . This contrasts with data of Weatherhead, et al (1982) who reported 50-200 plantlets produced per anther. However their data was obtained after a ten week culture period, with a subculture of excised anther callus after 3-4 weeks. Previous studies on African violet have indicated that an optimal response was obtained from anthers containing pollen in or near the first pollen division (Hughes, 1975). Work of Weatherhead, et al (1982) indicates that the response was confined to late uninucleate stage pollen. In this study it was found that Blue Rhapsody anthers cultured on Blaydes medium at the uninucleate, mitotic but also the binucleate stage of pollen development elicited an anther response. Furthermore, the maximum response with respect to callus and shoot induction was from anthers containing binucleate pollen. In direct contrast to the work of Weatherhead, et al (1982) it was found that the anthers of all 5 stages plated on MS medium supplemented with NAA:BAP 1:0.5mgl^{-1} produced callus. An optimal callusing response was produced from anthers containing uninucleate, mitotic and binucleate pollen.

Moreover, on this medium shoot production was greatest from stage 4 (mitotic) and stage 5 (binucleate) anthers.

Previous workers have cultured African violet anthers with the filament removed (Hughes, et al, 1975; Smith, et al, 1981). However results in this

study have shown that removing the filament causes damage to the anther leading to a 50% decline in anther response. Sunderland and Dunwell (1971) have shown that the presence of other parts of the flower whether free or attached) in the culture vessel does not affect the anther response, as long as, the anthers come into direct contact with the medium. Therefore, to optimise anther response in African violets it is recommended to culture anthers intact.

In agreement with Hughes, et al (1975) two types of callus were produced from African violet anthers namely: a creamy-brown callus that subsequently differentiated into shoots & roots, and a slow growing non-differentiating callus. As observed by Weatherhead, et al (1982), callus growth from African violet anthers cultured on the MS medium supplemented with NAA:BAP, 1:0.5mgl^{-1} was rapid and by 28d was in the linear phase of the growth cycle. At this period callus induction frequencies were determined. After a further two weeks on the same medium callusing from anthers declined and explants were either transferred to fresh medium or subcultured. Morphogenesis of African violet callus confirms the pattern of growth observed by Weatherhead, et al (1982). Thus the organisation of creamy-brown anther callus into nodular aggregates of cells, after six weeks of culture, is therefore the first necessary step to shoot primordia formation in such a culture system. Subsequently, vigorous plantlets could be raised and transferred to soil.

Furthermore, the production of adventitious shoots directly from the anther surface of anthers without an intermediary callus phase confirms the earliest reports of Hughes, et al (1975) and Smith, et al (1981). The formation of secondary adventitious shoot primordia, on the surface of the above shoots

is compatible with the data of Vazquez and Short (1978). The above workers observed large numbers of adventitious shoot buds, in the absence of callusing on ovary, sepal and petal tissue cultured on MS medium supplemented with BAP($1mgl^{-1}$) and NAA ($1mgl^{-1}$).

In-vitro culture of Saintpaulia ionantha cv Blue Rhapsody anthers on the medium of Weatherhead, et al (1982), causes a 57-70% loss in pollen viability after 28d. Similar results were reported by Sunderland and Wicks (1971) working with Nicotiana tabacum. Therefore, less than 20% of pollen grains from Saintpaulia anthers cultured initially at the critical period (late uninucleate - stage 3), as described by Weatherhead, et al (1982) for their medium survive 28d of incubation. Such a rapid degeneration of pollen grains, combined with the lack of any observable pollen calli or embryoids, leads to the conclusion that culturing Saintpaulia anthers onthe medium of Weatherhead, et al (1982) is not beneficial with respect to pollen viability and therefore subsequent sporophytic development of pollen.

Results from the work of Weatherhead, et al (1982) showed that callus was only produced from within anthers cultured on MS medium supplemented with NAA:BAP 1:0.5mgl^{-1}. However contrary to the above observations, histological and scanning electron microscopy studies, undertaken in this investigation revealed that 13-30% of anthers cultured exhibited callus formation from the anther interior(connective/ pollen tissue) but also the anther wall. Furthermore, the above investigations showed that anther callus originates predominately from the anther wall of cultured anthers. Therefore it is the division of the middle layer of cells, within discrete regions of the anther wall that is the prime cause of

rupturing observed at the anther surface (section 3.3.5.1 and Fig 3.6b). Evidence from isolated anther wall and connective/pollen tissue masses cultured on the medium of Weatherhead, et al (1982) further supports the above observations that anther wall tissue is more competent, with respect to callus induction, compared to tissue derived from the anther interior. Previous workers have shown that media rich in growth regulators encouarage the proliferation of tissue other than microspores (anther wall, connective and filament) and should be avoided. In such cases mixed calli with cells of different ploidy levels were obtained (Devreux, et al, 1971; Engvild, et al, 1972; Sunderland, et al, 1974).

At present the role of the haploid tissue in the connective/pollen tissue mass within African voilet anthers is undetermined. In this investigation no direct observations were made of pollen callus and/or embryoids, thus it is highly probable that any growth of the haploid microspores would be submerged by the profuse diploid connective callus. Therefore, to conclude it is evident that anther callusing on the medium of Weatherhead, et al (1982) is essentially a somatic phenomenon, with the callus derived from the anther wall or connective tissues.

Several investigations have demonstrated that the temperature under which donor plants are grown subsequently influences the androgenic response. Nitsch and Noreel (1973) found that Datura plants grown at 24°C exhibited a higher frequency of androgenesis (45%) than those grown at 17°C (8%). Furthermore, in Nicotiana knightiana anther response is doubled by increasing the donor plant growth temperature from 10°C to 20°C (Sunderland and Dunwell, 1977). In this investigation similar results were obtained.

A five fold increase of callus induction from anthers is found if Blue Rhapsody donor plant growth temperature is increased from 15 - 25°C. Growth of Blue Rhapsody plants at 15°C is inhibitory to androgenic response since 66% of plated anthers degenerated and 33% produced a non-morphogenic brown callus.

The beneficial effect of low temperature pretreatment of buds prior to culture was first demonstrated by Nitsch and Norreel (1972). Sunderland (1980) found such pretreatment useful in inducing pollen into a state of incipient embryogenesis prior to in vitro culture. Weatherhead, et al (1982) found that pretreating Saintpaulia buds at 7°C ijn the dark for 4,8 and 12d was inhibitory to callus production from the anther interior. Only a maximum of 5% of anthers produced callus after 4d and no callus production was observed after 8d. Obervations from this investigation confirm the results of Weatherhead, et al (1982). However, unlike the data of Weatherhead, et al (1982) 34% of anthers produced callus after 7d of chilling at 8°C in the dark. Therefore by increasing the chilling temperature by 1°C a seven fold increase in callus production was observed. Previous workers have shown that higher pretreatment temperatures may be more effective for some species (Sunderland and Wildon, 1979; Keller and Armstrong, 1979). When Blue Rhapsody anthers were cultured at 10°C for 14d,54%,produced callus. However the greatest response (95%) was obtained from anthers cultured at 15°C for 14d. Furthermore anthers cultured at 15/25°C (day/night alternation) induced significantly more callus compared to the 25°C control cultures. Cold treatment has been shown to delay senescence of anther somatic tissues (Pelletier and Henry, 1974) and rapid senescence is known to be inhibitory to the production of haploids (Pelletier and Ilami, 1972). However, continuous incubation at 15°C or 15°/25°C is detrimental to callus growth and morphogenesis which is always highest at 25°C. Therefore for future studies on African violet androgenesis

it is suggested that cultured anthers should be pretreated at 15°C for no more than14d then incubated at 25°C thereafter.

Previous studies have demonstrated that light does not appear to be necessary for the induction of androgenesis (Sangwan-Norreel, 1977; Sunderland and Roberts, 1977). Similar observations were made in this study for cultured Saintpualia anthers. The frequency of callus induction of anthers incubated in the dark was comparable to anthers cultured in the light.

Sugars are indispensible in the basal medium, as they are not the only source of carbon but are also involved in osmoregulation. In most species investigated the best results have been obtained with sucrose at a 2-3% concentration (Maheshwari, et al, 1980). Hughes, et al (1975), Smith, et al, (1981),Norris, et al (1982) and Weatherhead, et al, (1982) obtained adequate results when Saintpualia anthers were cultured on media containing sucrose at a 3% level. Comparable results were obtained in this study. Sucrose was essential for callus induction from Blue Rhapsody anthers and the optimum concentration required was 3%.

In the vastmajority of anther culture studies growth hormones have been found to be necessary for an androgenic response. The work of Weatherhead, et al (1982) demonstrated that solidified MS medium supplemented with NAA:BAP at 1:0.5mgl^{-1} resulted in a maximum response from Blue Rhapsody anthers Results from this investigation (testing three combinations of NAA:BAP) confirm the above workers earlier observations.

It is evident from this study that chelated iron in the medium of Weatherhead, et al (1982) is essential for callus induction from Blue Rhapsody anthers. This verifies previous reports that Iron (in a chelated form) and sucrose (see above) are the most important components of the medium (Vasil, 1980) with respect to callus induction.

The addition of activated charcoal to medium supplemented with hormones failed to enchance the frequency of anthers producing morphogenic callus. Although charcoal has increased the efficiency of androgenesis in tobacco (Anagnostakis, 1974; Bajaj, et al, 1977), anemone (Johansson and Eriksson, 1977), rye (Wenzel, et al, 1977) and potato (Sopory, et al, 1978) it does not seem to be effective for the anthers of Saintpaula ionantha. It seems likely that in plants like tobacco, charcoal absorbs inhibitory substances released from anthers and those present in agar (Wernicke and Kohlenbach, 1976) or in the medium itself. However, in Saintpaulia it is more probable that the level of growth substances (both endogenous and exogenous) is regulated by absorption into charcoal. This would explain the high level of non-morphogenic callus produced by anthers incubated onthe medium of Weatherhead, et al, (1982) containing 1% activated charcoal.

The addition of Polyvinylpolypyrrolidone (PVPP) to culture medium has recently been used to increase androgenesis in Datura innoxia (Tyagi, et al, 1981). However, no such observations were made of enhanced callus induction from Blue Rhapsody anthers when PVPP was added to the medium of Weatherhead, et al (1982).

Attempts to culture pollen which had been mechanically isolated from Blue Rhapsody anthers using the homogenization procedure of Nitsch (1974) in hanging drops proved to be unsuccessful. Previous workers have shown that the technique of pollen culture developed by Nitsch and co-workers, though successful with a wide range of species, often involves procedures which are unreliable and inefficient (Sunderland and Roberts, 1977). However, in this study only preliminary experiments were undertaken, covering a limited range of parameters. Therefore, until further investigations are carried out will it be possible to judge whether this technique is suitable for the culture of Saintpaulia pollen.

It is evident from the results that pretreatment of anthers at 8°C for 8d prior to culturing on liquid medium at 25°C has no adverse effect on the frequency of dehishence. Therefore attempts were undertaken to induce microspore division by adopting the pollen culture procedure of Sunderland and Roberts (1977). This involved pretreatment of buds at various temperatures for up to 28d, followed by culture on liquid medium (Nitsch, 1974). However this treatment proved to be highly ineffective. This confirms the earlier conclusion of Weatherhead, et al (1982) who reported that all anther cultures initiated on liquid medium failed to develop regardless of the composition of the culture medium.

Sharpe, et al (1972) induced the isolated pollen of Lycopersicon esculentum to form haploid callus using anthers as a nurse tissue. later Pelletier (1973) successfully induced androgenesis in Nicotiana tabacum microspores using Petunia callus as a nurse tissue. In this study it was observed that a maximum of 7.3% of uninucleate pollen grains produced multicellular pollen using anthers as a nurse tissue.

However, the multicellular pollen grains were only observed when the 'nurse' anther callus was in an differentiated state. Furthermore, it was not possible to determine whether the dividing pollen was derived from the pollen extract or the nurse anther. But in conclusion, it would be adverse not to consider using nurse tissue as a means of inducing embryogenesis in African violet pollen.

To summarise, it can be concluded that microsporogenesis in African violet follows the typical Angiosperm developmental pattern. Furthermore, as pollen development within an anther was relatively synchronous it was possible to correlate pollen stage with bud diameter. A dimorphic pollen population was observed in anthers excised from the oldest bud stage indicating that a maximum of 37% of pollen grains are potentially embryogenic. Various culture parameters were investigated and the following protocol is recommended to optimise callus induction production and subsequent morphogenesis from African violet (Blue Rhapsody) anthers. Donor plants should be grown at a constant temperature of 25^{o}C. Intact anthers containing pollen at the uninucleate-binucleate stage of development should be plated on the medium of Weatherhead,et al, (1982) incubated in the light at 15^{o}C (14d) then transferred to 25^{o}C for 28d. For maximum plantlet production anther-derived callus should be sub-cultured every 4-6 weeks and regenerated shoots (ca 1cm) transferred to shoot multiplication medium (Chapter 2, section 2.8.1). Thereafter shoots can be cultured in hormone free MS medium for rooting prior to transplanting.

Evidence from pollen viability, histology, SEM studies and from the culture of anther wall and connective/pollen tissue indicate that the majority

of anther-derived plantlets originate from somatic tissues. Preliminary studies indicate that multicellular pollen grains can be obtained using differentiated anther callus as a nurse tissue.

CHAPTER FOUR

ANALYSIS AND EVALUATION OF ANTHER-DERIVED AFRICAN VIOLET PLANTS

4.1 Introduction

Haploid plants derived from anther or microspore culture have potential use in mutation studies and breeding programmes (Nitsch, 1972; Reinert and Bajaj, 1977). The morphological, physiological or biochemical characteristics of haploids may make them valuable research tools (De Maggio, et al, 1971). An obvious phenotypic effect of haploidy is a decrease in plant or cell size. Nitsch (1972) found haploid tobacco plants to be a quarter the size of their parent. Smith, et al (1981) reported haploids of Saintpaulia ionantha Wendl to be two-thirds the size of the parent plant but they were identical in all phenotypic characteristics. However, Norris, et al (1982) reported phenotypic differences in haploid African violets. All haploids were much smaller than the parent lines the smallest was 33% (classed as a miniature) and the average was 64% of the size of the parent. Furthermore, phenotypic differences were recorded in leaf dimension (mature leaves), leaf indentation and shape were recorded. Studies of Smith, et al (1981) and Norris, et al (1982) showed that all haploid African violet plants flowered. The studies of Norris, et al (1982) revealed that the diameter of haploid flowers was significantly greater than that of the diploid parent. However, Smith, et al (1981) indicated that haploid African violets have a reduced number of anthers. Recently Bhaskaran, et al (1983) reported the production of an anther-derived miniature sterile African violet plant. To date no information has been obtained on flower form and the numbers of flower stalks and flowers produced by anther-derived plants compared to the parent line.

The ploidy level of plants can only be determined unequivocally by means of Karyotypic analysis.

However a more rapid method can be used which is reliant on the number of chloroplasts found in the stomatal guard cells. In potatoes and cotton plants a relationship was found between the number of plastids in the guard cells and the number of chromosomes. Frandsen (1967) observed 12, 16 and 22 plastids respectively in the stomata of haploid, triploid and tetrapolid potatoes. Chaudhari and Barrow (1975) identified cotton haploids accurately by the chloroplast-count technique and concluded that the stomatal chloroplst-count technique is rapid and accurate for classifying haploid plants at any stage of growth, as long as the leaves are living at the time of examination. This technique has been used by Bhaskaran, et al (1983) as a preliminary screen for haploidy amongst a population of anther-derived African violet plants. The above workers found that the number of chloroplasts in the guard cells was comparable to the parent plants in two of thier anther-derived lines. However, their third and fourth lines had respectively significantly 51% more and 26% less chlorplasts in their guard cells.

Callus cultures have been shown to be a rich source of genetic variability (D'Amato, 1978; Skirvin, 1978). However by using anther culture it is possible that not only haploids but plants of various ploidy levels including mutants and chimeras could be regenerated (McComb, 1977). A wide range of genetic variability has been reported for a number of species that exhibit variation in chromosome number of anther-derived callus, protoplasts and plants (Bajaj, 1983). For example the anther-derived lines of Arachis hypogaea (Bajaj, et al, 1981), Barassica napus (Keller and Armstrong, 1977) and Oryza sativa (Chen and Chen, 1980) showed a wide range of genetic variability, as the chromosome number varied from haploid to octoploid with abundant anenploids. Previous reports

of chromosome counts of anther-derived African violet plants at the haploid level have ranged from N=13 to N=15 (Hughes, et al, 1975; Smith, et al, 1980; and Norris, et al, 1982). Recent work by Bhaskaran, et al (1983) showed that their anther-derived plants were either diploid or tetraploid. However the above workers did not preclude a haploid origin for their anther-derived plants as an earlier root tip ploidy determination established them to be haploids. Furthermore the occurence of only diploids or tetraploids and no triploids and pentaploids, gives strong support for the spontaneous doubling of haploids by endomitosis.

4.2 Aims and Experimentation

The aim of this study was to evaluate plants regenerated from anther-derived cell lines.

Twenty Blue Rhapsody anther donor plants were derived from leaf cuttings (Chapter 2, section 2.1.1.). Three anther-derived lines were regenerated from anther explants cultured on the media of Blaydes (1966). A further 107 plant lines were regenerated from anthers cultured on the medium of Weatherhead, et al (1982) supplemented with various levels of sucrose and hormones (Chapter 3). Plants were regenerated by transferring ca 1cm high shoots excised from anther explants at 4-8 leaf stage, to hormone free MS medium (Chapter 2, section 2.7). After about five weeks of culture on hormone-free rooting medium, young African violet plantlets were transferred to Jiffy-7 peat pellets then eventually ttansplanted to potting compost and grown under greenhouse conditions (Chapter 2, section 2.9).

A survey of both the vegetatively propagated parent and the anther derived lines were made six

months after establishment in compost. The range of morphological characters assessed included rosette diameter, leaf size, leaf margins, flower stem number, number of flowers and flower buds, flower form and anther number (Chapter 2, section 2.11.2).

The ploidy level of plants under investigation was determined by karyotypic analysis and by the number of chloroplasts in stomatal guard cells (Chapter 2, section 2.11.3). Chromosome counts were made on root tips of leaf cuttings form all parent and anther-derived plants. Chloroplast numbers were determined of at least 25 stomata of parental and anther-derived plantlets. All plants which on the basis of morphological and ploidy assessments referred to above appeared to be haploid were then subject to a chromosome check of the shoot meristem.

4.3 Results

4.3.1 Morphological Analysis

Rosette diameter - The mean plant diameter appears to be one of the best criteria for size increase or reduction in anther-derived lines of African violet (Table 4.1 and Fig 4.1). The majority of plants exhibited a significant increase in size ranging from 9% to 33% of the diameter of the parent plants. However, three plants, designated as the non-chimera, unstable chimera (both regenerated from the same anther callus (Fig 4.2) and miniature (Fig 4.3) were significantly smaller than the parent line (Table 4.1). The size reduction ranged from 12-51% of the diameter of the parent plant.

Leaf-size - Mature leaf dimensions confirmed the overall size increase recorded for the majority of anther-derived plants (Table 4.1). The non-chimera, chimera and miniature plants showed a significant reduction in leaf size of an average 9-50% respectively.

Fig. 4.1 Comparison of a Blue Rhapsody anther-donor parent plant (top) with an anther-derived plant (bottom) (x0.6).

Table 4.1 Comparison of Vegetative Characteristics of Blue Rhapsody Parent with Anther-Derived Lines

Parent and Anther-Derived Lines		Number of Plants Assessed	Plant Diameter (cm)	Leaf Size (cm)		Leaf Margins (%)	
				Length	Width	Entire	Crenate-fine
Parent Lines (Blue Rhapsody)		20	19.5±0.5	5.2±0.1	4.6±0.1		100
Anther-Derived Lines							
Blaydes's Medium		3	22.6±2.2	5.4±0.1	4.9±0.1		100
Modified Media of Weatherhead, et al (1982)							
Surcrose (%)	3%	13	21.3±1.1	5.5±0.1	5.0±0.1	7.7	92.3
	6%	17	23.6±0.9	5.7±0.1	5.1±0.1		100
	9%	12	25.6±1.3	5.5±0.1	5.0±0.1	50	50
	12%	12	25.7±0.8	5.5±0.1	5.3±0.1		100
	15%	11	24.0±1.2	5.7±0.1	4.8±0.1		100
NAA:BAP(mgl^{-1})	1:0.5	13	23.0±1.5	5.5±0.2	5.2±0.2		100
	1:1	11	26.0±1.3	5.8±0.2	5.3±0.2		100
	0.5:1	15	23.0±0.8	5.5±0.2	5.0±0.1		100
Non-chimera		1	17.0	4.7±0.1	4.4±0.2		100
Unstable chimera		1	15.0	3.3+0.1	3.1+0.1		100
Miniature	1	9.5	2.3±0.1	2.6±0.2		100	

Parent lines were derived from leaf cuttings. Anther-derived lines originate from anthers cultured on either Blaydes's medium (section 3.3.4) or the medium of Weatherhead, et al (1982) (supplemented with various levels of sucrose (section 3.3.12) and hormones (section 3.3.13). Plants derived from adventitious shoots observed arising directly from the anther surface (section 3.3.5.2) are included in the 3% sucrose data. Non-chimera, chimera and miniature plants were derived from anthers cultured on the medium of Weatherhead, et al (1982). Plants were grown under greenhouse conditions and assessed after establishment in comppost for six months. Mean and standard errors are indicated.

Fig. 4.2.a: Non-chimera (left) and unstable chimera (right) plants regenerated from the same anther callus (x0.4) with corresponding leaves.(b: adaxial and abaxial surfaces; x0.8).

Fig. 4.3.a: Comparison of anther-derived plant (left) and anther-derived miniature (right). (x0.4). b: Anther-derived miniature (x1.1).

Leaf margins - Leaf margins of the anther-derived lines showed little variation when compared to the parent plants. Only 6% of anther-derived plants showed any variation having an entire leaf margin, whereas, the majority of plants displayed the same finely crenate margin of the Blue Rhapsody parent (Table 4.1).

Flower stalk number - The mean number of flower stems was similar among the population of anther-derived lines compared to the parent plants (Table 4.2). However, both the non-chimera and unstable chimera produced half as many flower stalks when compared to the anther donor plants. The minature was the only anther-derived plant that did not produce any flower stalks.

Number of flowers and flower buds - The mean number of flowers and flower buds produced did not vary significantly amongst the population of anther-derived plants compared to the parent plant line. Again, both the non-chimera and chimera lines produced significantly less flowers when compared to the parent line. Furthermore no flowers were produced by the anther-derived miniature.

Flower diameter - All anther-derived plants exhibited a significant reduction in mean flower diameter ranging from 6.7% to 28.6% (Table 4.2). The greatest reduction was obtained by plants that were regenerated from anther callus cultured on the medium of Weatherhead, et al (1982) supplemented with NAA:BAP, 1:1mgl^{-1} (Table 3.19)

Flower form - Flower form was the most variable characteristic measured (Table 4.2). Whereas all parent plants had single flowers only 24 (22%) anther-derived plants regenerated true to type. Of the

Table 4.2 Comparison of Floral Characteristics of Blue Rhapsody Parent with Anther-Derived Lines

Parent and Anther-Derived Lines		Number of Plants Assessed	Flower Stalk Number	Number of Flowers and Flower Buds	Flower Diameter (cm)	Flower Form Single	Semi-Double	Dble	Anther Number
Parent Lines (Blue Rhapsody)		20	8.6±0.6	41.2±11.2	35.3±0.6	100			5
Anther-Derived Lines									
Blaydes's Medium		3	6.6±1.2	35.0±7.8	32.4±1.6	33.3	66.7		4.0±1
Modified Media of Weatherhead, et al (1982)									
Sucrose(%)	3%	13	8.0±0.8	39.3±13	30.0±1	38.4	23.2	38.4	5±1
	6%	17	8.3±1.0	43.5±9.4	32.9±1.1	23.5	64.7	11.7	6.0±1
	9%	12	8.3±3.3	43.8±15.2	31.8±2.2	50	50		4.0±2
	12%	12	6.5±0.5	37.3±3.9	30.3±1.1		50	50	7.0±1
	15%	11	8.0±0.9	44.5±5.5	32.3±1.5		100		6.0±1
NAA:BAP(mgl^{-1})	1:0.5	13	6.3±1.9	32.5±8.4	26.4±1.1		100		4.0±2
	1:1	11	8.7±0.7	39.3±3.4	25.2±0.3		100		6.0±1
	0.5:1	15	7.8±0.7	39.0±7.3	26.5±0.8	40	20	40	4.0±2
Non-chimera		1	4.0	25.0	26.7±2.4	100			5.0±1
Unstable chimera		1	4.0	21.0	26.4±1.9	100			7.0±1
Miniature		1	0	N/A	N/A	N/A			N/A

Parent lines were derived from leaf cuttings. Anther-derived lines originate from anthers cultured on either Blaydes's medium (Section 3.3.4) or the medium of Weatherhead, et al (1982) (supplemented with various levels of sucrose (section 3.3.12) and hormones (section 3.3.13). Plants derived from adventitious shoots observied arising directly from the anther surface (section 3.3.5.2) are included in the 3% sucrose data. Non-chimera, chimera and miniature plants were derived from anthers cultured on the medium of Weatherhead, et al (1982). Plants were grown under greenhouse conditions and assessed after establishment in compost for six months. Mean and standard errors are indicated.

remainder, 66 (60.5%) produced semi-double and 19 (17.4%) developed double flowers respectively.

Anther number - All anther deived plants showed a variation in the number of anthers per flower when compared to the parent line. (Table 3.23). The variation of mean number of anthers per flower ranged from a 20% decrease to a 40% increase.

4.3.2 Analysis of Guard-Cell Chloroplasts

Chloroplast numbers in guard cells were comparable in the parent plants and in the majority of anther-derived plants 'Table 4.3, Fig 4.4). However, chloroplast numbers were significantly lower in the miniature whem compared to the parents.

4.3.3 Karyotypic analysis

Chromosome counts were made on root tips of leaf cuttings of tissue culture derived plants. Leaf cuttings from conventionally raised parent plants of comparable age were used as a control. Although the parent plants had the diploid set of chromosomes (n=28), the anther-derived plants were either diploid or aneuploid (n=29) (Table 4.3, Fig 4.4). The mean root and shoot meristem chromosome counts for the miniature plant were 29.0 ± 0.4 and 28.6 ± 0.5 respectively.

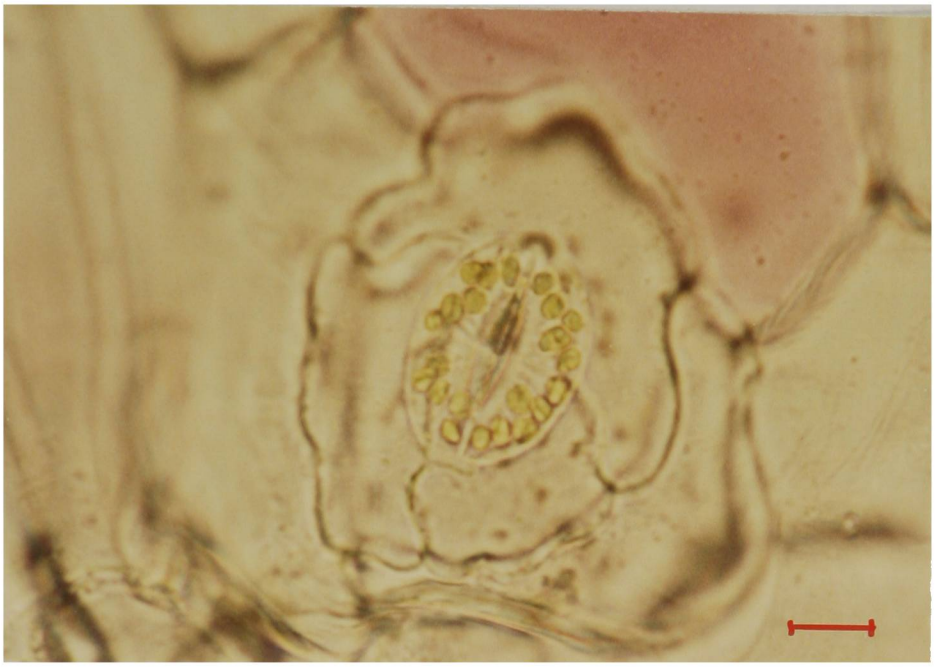
4.4 Discussion

The data reported in this study presents a comparison of African violet plants derived from anther culture with parent plants produced from leaf cuttings. Previous workers have reported a decrease in rosette diameter, leaf length and width in anther-derived plant lines (Smith, et al, 1981, Norris, et al, 1982). In this investigation three lines (non-chimera, unstable chimera and miniature) showed a signficant reduction in plant

Table 4.3 Comparison of Guard-Cell Chloroplast Numbers and Root-tip Chromosome Counts of Blue Rhapsody Parent with Anther-Derived Lines

Parent and Anether-Derived Lines		Number of Plants Assessed	Guard Cell Chlorplast Numbers	Root-tip Chromosom Counts
Parent Lines (Blue Rhapsody)		20	18.6 ± 0.6	28.3 ± 0.5
Anther Derived Lines				
Blayden's Medium		3	19.4 ± 0.8	28.5 ± 0.9
Modified Media of Weatherhead et al (1982)				
Sucrose(%)	3%	13	19.7 ± 1.0	28.5 ± 0.5
	6%	17	20.5 ± 0.7	28.9 ± 0.3
	9%	12	17.7 ± 1.6	28.8 ± 0.4
	12%	12	18.1 ± 0.5	28.4 ± 0.6
	15%	11	18.4 ± 0.4	29.0 ± 0.6
NAA:BAP (Mgl^{-1})	1:0.5	13	19.9 ± 1.3	29.5 ± 1.0
	1:1	11	20.4 ± 0.5	29.5 ± 0.6
	0.5:1	15	19.7 ± 1.7	29.0 ± 0.7
Non-chimera		1	21.3 ± 0.7	28.6 ± 0.3
Unstable Chimera		1	21.5 ± 0.4	28.5 ± 0.6
Miniature		1	11.0 ± 0.4	28.4 ± 0.4 (28.6 ± 0.5)

Guard cell chloroplast numbers were determined from young and mature African violet leaves. Chlorplasts from the guard cells of 25 stomates were counted from each leaf. For root tip chromosome counts the first countable, intact cell encountered from each of ten separate fields of view was counted. A minimum of 2 root tips were counted per plant. Shoot meristem chromosome counts for the miniature African violet are shown in parenthesis. Mean and standard errors are indicated.



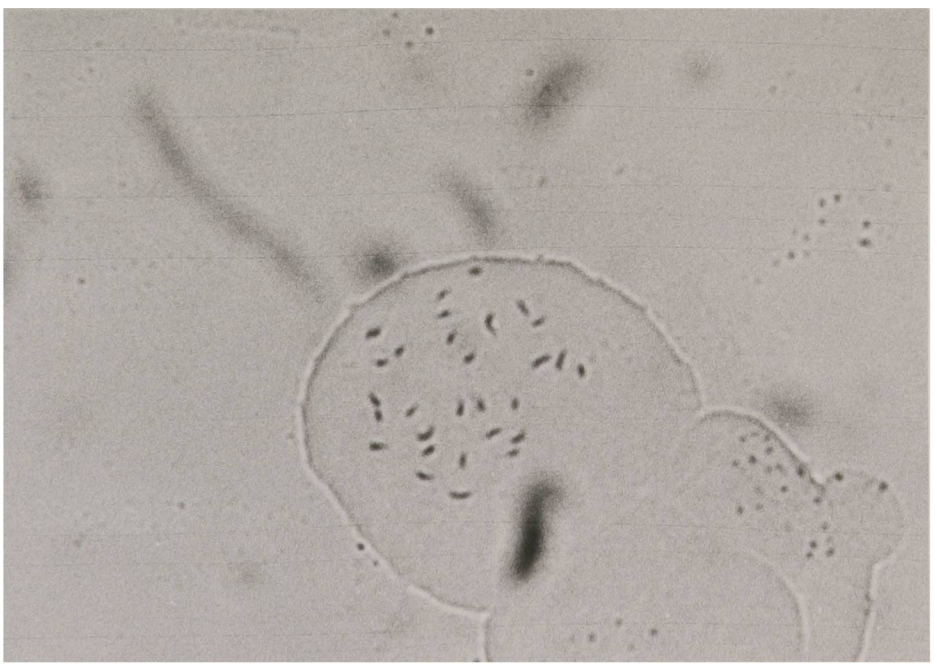


Fig. 4.4.a: Stoma, with 22 guard cell chloroplasts on the lower epidermis of an anther-derived leaf. Bar = 10µm.
b: Feulgen-stained root tip cell from an anther-derived plant showing the chromosomes (n=29)(x 3320).

diameter. The greatest reduction was observed by the miniature plant (51%) indicating that this plant is probably haploid. However the majority of lines showed a significant increase in these three characteristics. A recent report has indicated that African violets derived from the culture of somatic tissue have improved vegetative characteristics compared to plants propagated by conventional means (Cassells and Plunkett, 1984). Consequently it is highly probably that the majority of anther-derived plants are derived from the somatic tissue of the anther (Chapter 3, section 3.3.5). In this study 6% of anther-derived plants had an entire leaf whereas the parent plants had a finely crenate leaf margin. This confirms the earlier work of Norris, et al (1982) who also observed variations in the leaf margin of anther-derived African violet plants.

The majority of anther-derived plants flowered confirming the earlier observations of Smith, et al (1981) and Norris, et al (1982). However, the miniature plant did not flower. Bhaskaran, et al (1983) reported a similar lack of flowering from a miniature anther-derived African violet plant. As monohaploids are generally sterile (Nitsch, 1972) this adds further evidence to the possibility that the miniature is a haploid. Flower stalk, flower and flower bud production in most of the anther-derived lines was comparable to the results obtained from the conventionally propagated anther donor parent lines. Only two plants (non-chimera and unstable chimera) produced significantly less flower stalks, flowers and flower buds. However in general this work confirms the conclusion of Cassells and Plunkett (1984) that flower production is not influenced by tissue culture of African violet contrary to previous observations (Norris, et al 1982) all anther-derived polants regenerated in this study showed a significant decrease in the flower diameter. Furthermore only 22% of anther-

derived lines regenerated produced single flowers. The remaining lines produced semi-double and double flowers. Variation in the number of petals has been reported from plants derived from anther culture of Geranium (Abo El-Nil and Hilderbradt, 1972). The double flower mutation has been found to be dominant to the wild type in Saintpaulias (Reed, 1954). Homozygotes (Doubles) are detectable from the heterozygotes (semi doubles) as they have many more petals arranged in a more orderly fashion. Furthermore, the flowers of the doubles are much smaller than those of the semi-doubles and wild types (Reed, 1954). Therefore, it is highly probably that the decrease in flower diameter observed in this study for the majority of anther-derived plants can be accounted for by the high frequency of double and semi-double mutant flowers. The reduction in flower diameter observed in the non-chimera and unstable chimera is possibly due to the decreased vigour of these plant lines (see above).

Previous workers have shown that anther-derived plants have a reduced number of anthers (smith, et al, 1981). Work in this study has shown that the number of anthers per flower in anther-derived lines can vary above and below the parental number of five.

Sugura (1936) published the first cytological study of Saintpaulia and reported the 2M chromosome number to be 28. Later workers have recorded a similar number (Espino, et al, 1981). However African violet chromosomes are small and tend to stick together (Sugiura, 1936) and some variations in numbers have been reported. Studies by Wilson (1951) and Ehrlich (1958) indicated a Zn=30 for Saintpaulia. Parent plant lines in this investigation showed a diploid count of Zn=28. The results on ploidy determination indicate that the anther-derived plants were either diploid or aneuploid (Zn=29). Khokar (1983) reported

a vigorous plant type, derived from tissue culture, that was aneuploid. Therefore it is possible that some of the increase in morphological variation reported in this study is due to aneuploidy. Evidence from the chloroplast numbers in the stomatal guard cells support the above view as the numbers were comparable to that of the diploid parent line. Furthermore, chloroplast numbers were similar to those reported by Bhaskaran, et al (1983) for diploid African violets obtained by anther culture. However this does not preclude a haploid origin for the miniature plant. Since phenotypically it is a true miniature and it has a reduced number of chlorplasts inthe guard cells, both support its haploid origin. Since reduction and chlorplast counts in guard cells (Chaudhari and Barrow, 1975) have been used as reliable methods for the determination of ploidy in plants. Also the miniature did not flower indicating a haploid origin. Furthermore, because of the slow growth exhibited by the miniature it is possible that spontaneous doubling had occured inthe interval between the twomethods of determining ploidy levels. Spontaneous doubling of haploids is encountered in tissue cultures by endomitosis (Vasil, 1980). Previous workers from different laboratories have reported doubling of their tissue culture derived, African violet, haploid plants. (Hughes pers comm, 1983; Smith pers comm, 1983).

CHAPTER FIVE

EFFECTS OF TEMPERATURE AND PHOTOPERIOD ON GROWTH OF AFRICAN VIOLETS

5.1 Introduction

Commercialy grown African violets require temperatures of 20-25°C for optimal growth and flowering (Herklotz, 1968). However, this requirment at a time of rising fuel costs is causing considerable concern amongst northern commerical growers. Furthermore even with present energy conserving measures (thermal curtains, double glazing, etc) many nurseries fear closure if heating costs in one form or another are not drastically reduced. (Bilkey, 1981). Recently, the use of low temperature tolerant genotypes (Bilkey, 1981) have been used in an attempt to reduce the cost of large scale production of Saintpaulias. However, work by Went (1959, 1960) has shown that it is possible to grow African violets, and other plant species, at lower temperatures without altering the parent genotype.

In a series of experiments Went (1960) found that the autonomous circadian rhythm (internal rhythm) is so ingraved in plants that they are unable to adjust themselves to an external daily-rhythm. Furthermore, it was shown that their internal rhythm had a termperature co-efficient of about 1.2 and that the length of circadian rhythms can be seen as a function of temperature. Went (1960) demonstrated this to Tomato, Baeria chryostoma, Saintpaulia ionantha and Begonia. This work was confirmed in the same year by Keterllapper (1960) for peanuts and tomatoes.

Went (1960) grew plants in enviromental cabinets, in which both temperature and light/dark cycle length could be regulated independently of each other. To make all his experiments strictly comparable, all cycles were equally divided into half light and half dark periods, eg 24h cycle; 12h light/12h dark. Furthermore, light intensity was kept constant at 500 ftc. The results showed that at any one

temperature a particular cycle length gave optimal growth (known as optimal cycle length), whereas both at shorter and larger cycle lengths growth was diminished, compared with the optimal cycle length. At the optimal growing temperature, this cycle clength was 24h (12h light: 12h darkness), but sets of plants grown at other temperatures showed differences in optimal cycle lengths. Generally at higher temperarures the optimal length was less than 24h, whereas at lower temperatures this length was greater. Therefore, tropical plants such as African violets, cannot survive at low temperatures as their circadian rhythm is synchronized with the 24h external cycle at a high temperature ($25^{o}C$) (Went, 1960). Furthermore, Went (1960) showed that African violets grown at $15^{o}C$ on a 24h and 33h cycle had deteriorated whereas, on a 29h cycle length, they were vigorous and flowering. African violets did not survive exposure to $10^{o}C$ when maintained on a 24h cycle, but when subjected to a 32h cycle length they tolerated this low temperature.

5.2 Aims and Experimentation

The aims of this chapter were as follows:-

1. To test plants derived from three African violet cell Lines (Blue Rhapsody parent and anther-derived lines and the cold tolerant variety, Endurance (Fig 5.1) for low temperature tolerance.
2. To evaluate whether growing the above three lines under optimal (light/dark) cycle lengths as described by Went (1960) is beneficial to plant growth and development.

To satisfy the above aims plants were grown at $10\pm1^{o}C$, $15\pm1^{o}C$ and $25\pm1^{o}C$ under various light/dark cycles. Light was supplied to 8 week old plants established from a leaf cutting, at a intensity similar to that described in Wents (1960) experiments ($60\mu mol\ m^{-2}s^{-}1$), (Chapter 2, section 2.10.2).

Fig 5.1 Mature flowering plant of a commercially available cold-tolerant line Endurance var Wilson (x0.5)

The photoperiod was either 16h light followed by 8h darkness (referred to in the text as a 16h:8h cycle) or that of the optimal cycle length (the particular cycle length that gave optimal growth at any given temperature). As in Wents (1960) work to make all experiments strictly comparable, all cycle elngths were divided equally into half light and half dark periods (Fig 5.1). Therefore at $10^{o}C$, $15^{o}C$ and $25^{o}C$ the respective optimal cycle lengths were; 16h:16h (32h cycle); 14h30min:14h30min (29h cycle) and 11h30min:11h30min (23h cycle) of alternating light and dark periods.

All the above treatments were compared to plants grown under glass at $25\pm2^{o}C$ during the period May - July 1984.

Eight week old plants, of the three African ciolet lines under investigation were characterised before being used in the experiment (Table 5.1). Subsequently growth and flowering data was measured after 6 and 12 weeks of treatment. For each plant the following parameters were recorded: plant diameter Tables 5.2, 5.5 and 5.8) leaf (Tables 5.3, 5.6 and 59.) and flower stalk number (Tables 5.4, 5.7 and 5.10) and the frequency of chlorosis (Table 5.11). At the start of the experiment and after 12 weeks, plants were harvested and the total dry weights of leaves/shoot, roots and flower stalks were determined separately, with an average in each case being taken of at least 10 plants in each treatment (Tables 5.12, 5.13, 5.14 and Figs 5.2, 5.3, 5.4 and 5.5).

Thereafter comparisons of plant growth under the various light and temperature regimes were made within and between the three African violet lines investigated.

Table 5.1 Growth of Three African Violet Lines Established from Leaf Cuttings

	Blue Rhapsody	Anther-Derived	Endurance
Plant Diameter (cm)	10.8±0.21	10.6±0.27	10.5±0.30
Leaf Number	11.2±0.17	11.9±0.3	12.3±0.24
Leaf Length (cm)	3.3±0.04	3.7±0.05	3.7±0.1
Leaf Width (cm)	3.2±0.04	3.7±0.05	3.5±0.05
Leaf/Shoot Biomass(g)	0.52±0.03	0.54±0.05	0.58±0.08
Root Biomass(g)	0.15±0.01	0.13±0.02	0.17±0.07

Growth characteristics of 8 week old plants from three African violet lines. Plants were derived from plantlets selected from 2 month old leaf cuttings. Data collected from 70-100 plants from each line. Leaf/shoot and root biomass data taken from a sample of 10-15 plants. Mean and standard errors are indicated.

5.3 Results

5.3.1 Blue Rhapsody Plants

Data represented in Tables 5.2 (plant diameter), 5.3 (leaf production) and 5.12 (leaf/shoot biomass) show essentially the same pattern of growth for Blue Rhapsody at 10°C, 15°C and 25°C under the 16h:8h, light:dark cycle and the three optimal cycle lenghts (10°. 16h light:16h dark; 15°,14h30min light: 14h30min dark; 25°,11h30min light:11h30mindark).

The greates growth rate was observed by plants grown at 25°C under both the 23h (11h30min:11h30min) and 16h:8h, light:dark cycles. On lowering the growing temperature to 15°C the incidence of chlorosis increases (Table 5.11) and the leaf/shoot biomass production decreases (Table 5.12). Chlorotic symptoms were always significantly higher on plants grown under the extended cycle lengths irrespective of the temperature plants were grown at (Table 5.11). Although 25-39% of leaves from all Blue Rhapsody plants grown at 15°C showed some chlorotic symptoms (Table 5.11, Fig 5.2) plants still exhibited a two fold increase in dry weight after 12 weeks of growth (Table 5.12). However, plants grown at 10°C displayed an 8-15% decrease in leaf/shoot biomass. Furthermore, the chlorotic symptoms were most pronounced on plants at 10°C under the extended 32h (16h:16h) light:dark cycle (Table 5.11).

Plants grown at 10°C exhibited extensive root senescence, causing the significant 40-70% decline in root biomass observed. Root growth was greatest at 25°C under both the 16h:8h and optimal cycle lengths (Table 5.13).

Tables 5.2-5.14 inclusive and Figures 5.2, 5.3, 5.4 and 5.5.

Effects of temperature and photoperiod on vegetative and floral characteristics of young established African violet plants.

Three lines, parent line and anther-derived line of African Violet (var Blue Rhapsody) and an existing cold-tolerant variety endurance were incubated at $10\pm1^{o}C$, $15\pm1^{o}C$ and $25\pm1^{o}C$. Light was supplied by 36W fluorescent tubes at an intensity of $60uEm^{-2}s^{-1}$ (10cm from the plants). The photoperiod was either 16h light followed by 8h darkness or that of the optimal cycle length which was dependent on the temperature plants were grown ($10^{o}C$, 16h light:16h darkness; $15^{o}C$, 14h30min light: 14h30min darkness; $25^{o}C$, 11h30min light:11h30min darkness). (After F W Went, 1960, see 4.2). All the above treatments were compared to plants grown undr glass at $25\pm2^{o}C$ during the months of May, June and July 1984. The following characteristics were measured for each plant at day 0, week 6 and/or week 12:plant diamter (Tables 5.2, 5.4, 5.8), leaf and flower stalk numbers (Tables 5.3, 5.6, 5.9 and 5.4, 5.7, 5.10 respectively) and the frequency of chlorosis (Table 5.11). At day 0 and after 12 weeks plants were harvested and the total dry weights of leaf/shoot, roots and inflorescences were determined separately, with an average in each case being taken of at least 10 plants in each treatment (Tables 5.12, 5.13 and 5.14. Standard errors are indicated.

Table 5.2 Effect of Temperature and Photoperiod on Growth of Blue Rhapsody Plants

	Photoperiod (light:dark; h, min)	Growing Temperature (^{o}C)	Plant Diameter (cm) Day 0	Wk 6	Wk 12	Percentage Increase/ Decrease in Plant Diamter
Optimal Cycle Lengths	16:8	$10\pm1^{o}C$	10.7 ± 0.3	10.5 ± 0.6	10.4 ± 0.8	-2.8
	16:8	$15\pm1^{o}C$	10.3 ± 0.6	13.0 ± 0.8	15.1 ± 0.9	46.6
	16:8	$25\pm1^{o}C$	11.1 ± 0.6	14.4 ± 0.6	17.5 ± 0.7	57.6
	16:16	$10\pm1^{o}C$	9.7 ± 0.8	11.3 ± 0.6	10.8 ± 1.1	10.0
	14.30:14.30	$15\pm1^{o}C$	9.8 ± 0.5	13.2 ± 0.5	14.9 ± 0.6	52.0
	11.30:11.30	$25\pm1^{o}C$	10.8 ± 0.7	13.3 ± 0.8	16.7 ± 0.7	54.6
	Greenhouse Controls	$25\pm2^{o}C$	10.6 ± 0.5	14.2 ± 0.8	16.6 ± 0.8	37.7

Table 5.3 Effect of Temperature and Photoperiod on Leaf Production of Blue Rhapsody Plants

	Photoperiod (light:dark; h, min)	Growing Temperature (°C)	Leaf Production Day 0	Wk 6	Wk 12	Percentage Increase/ Decrease in Leaf Numbers
Optimal Cycle Lengths	16:8	10±1°C	11.3±0.5	11.7±0.8	9.4±0.8	-16.8
	16:8	15±1°C	11.1±0.2	14.8±0.8	15.0±1.0	35.1
	16:8	25±1°C	11.4±0.4	14.5±0.7	17.9±1.2	57.0
	16:16	10±1°C	11.9±0.7	10.7±0.8	9.4±1.3	-21.0
	14.30:14.30	15±1°C	11.2±0.5	16.1±1.1	17.7±1.1	58.0
	11.30:11.30	25±1°C	10.8±0.6	16.2±0.7	19.9±1.4	84.3
	Greenhouse Controls	25±2°C	10.7±0.3	12.4±0.8	16.9±0.7	57.9

Table 5.4 Effect of Temperature and Photoperiod on Flower Stalk Production of Blue Rhapsody Plants

	Photoperiod (light:dark, h, min)	Growing Temperature (°C)	Flower Stalk Number Day 0	Wk 6	Wk 12
	16:8	10±1°C	0	0	0
	16:8	15±1°C	0	0.6±0.4	1.8±0.5
	16:8	25±1°C	0	2.2±0.6	3.6±0.8
Optimal Cycle Lengths	16:16	10±1°C	0	0	0
	14.30:14.30	15±1°C	0	1.5±0.5	2.0±0.5
	11.30:11.30	25±1°C	0	1.5±0.3	2.3±0.7
Greenhouse Controls		25±2°C	0	2.0±0.3	2.8±0.4

Table 5.5 Effect on Temperature and Photoperiod on Growth of Anther-Derived Plants

	Photoperiod (light:dark, hr, min)	Growing Temperature (^{o}C)	Plant Diameter (cm) Day 0	Wk 6	Wk 12	Percentage Increase/ Decrease in Plant Diameter
	16:8	10±1^{o}C	9.7±0.2	9.2±0.8	8.9±0.8	-8.9
	16:8	15±1^{o}C	10.0± 0.4	14.4±0.6	20.5±0.5	105.0
	16:8	25±1^{o}C	10.4±0.5	14.5±0.5	20.3±0.6	95.1
Optimal Cycle Lengths	16:16	10±1^{o}C	10.1±0.3	10.9±0.6	8.5±0.7	-15.8
	14.30:14.30	15±1^{o}C	10.5±0.4	15.6±0.5	17.6±0.4	67.6
	11.30:11.30	25±1^{o}C	11.2±0.7	15.4±0.7	19.4±0.5	73.2
Greenhouse Controls		25±2^{o}C	10.7±0.6	14.7±1.0	19.7±0.7	84.1

Table 5.6 Effect of Temperature and Photoperiod on Leaf Production of Anther-Derived Plants

	Photoperiod (light:dark, h, min)	Growing Temperature (^{o}C)	Leaf Production Day 0	Wk 6	Wk 12	Percentage Incr Decrease in Lea Numbers
	16:8	$10\pm1^{o}C$	11.0 ± 0.4	10.0 ± 0.5	12.9 ± 1.5	17.3
	16:8	$15\pm1^{o}C$	12.0 ± 0.7	16.0 ± 1.0	26.0 ± 1.7	116.7
	16:8	$25\pm1^{o}C$	11.0 ± 0.3	15.0 ± 0.3	21.0 ± 1.1	90.9
Optimal Cycle Lengths	16:16	$10\pm1^{o}C$	11.0 ± 0.4	13.0 ± 0.6	13.0 ± 0.9	15.3
	14.30:14.30	$15\pm1^{o}C$	11.0 ± 0.4	15.0 ± 1.1	21.0 ± 1.5	90.9
	11.30:11.30	$25\pm1^{o}C$	12.0 ± 1.3	16.0 ± 1.5	20.0 ± 1.4	66.6
	Greenhouse Controls	$25\pm2^{o}C$	12.0 ± 0.8	15.0 ± 0.9	23.0 ± 2.6	91.7

Table 5.7 Effect of Temperature and Photoperiod on Flower Stalk Production of Anther-Derived Plant

	Photoperiod (light:dark, h, min)	Growing Temperature (^{o}C)	Flower Stalk Production Day 0	Wk 6	Wk 12
	16:8	$10\pm1^{o}C$	0	0	1.7 ± 0.5
	16:8	$15\pm1^{o}C$	0	0.5 ± 0.2	4.3 ± 0.4
	16:8	$25\pm1^{o}C$	0	2.5 ± 0.3	5.0 ± 0.5
Optimal Cycle Lengths	16:16	$10\pm1^{o}C$	0	0.3 ± 0.2	0.6 ± 0.2
	14.30:14.30	$15\pm1^{o}C$	0	1.6 ± 0.3	2.6 ± 0.5
	11.30:11.30	$25\pm1^{o}C$	0	1.7 ± 0.3	2.7 ± 0.3
	Greenhouse Controls	$25\pm2^{o}C$	0	2.1 ± 0.1	4.1 ± 0.1

Table 5.8 Effect of Temperature and Photoperiod on Growth of Endurance Plants

	Photoperiod (light:dark, h, min)	Growing Temperature (^{o}C)	Plant Diameter (cm) Day 0	Wk 6	Wk 12	Percentage Increa Decrease in Plant Diameter
	16:8	$10\pm1^{o}C$	10.5 ± 0.3	15.3 ± 0.5	17.3 ± 0.7	66.6
	16:8	$15\pm1^{o}C$	10.8 ± 0.5	17.7 ± 0.5	24.1 ± 0.5	123.1
	16:8	$25\pm1^{o}C$	$98.\pm0.3$	15.8 ± 0.4	21.2 ± 0.3	116.3
Optimal Cycle Lengths	16:16	$10\pm1^{o}C$	10.8 ± 0.2	13.5 ± 0.2	14.5 ± 0.5	34.2
	14.30:14.30	$15\pm1^{o}C$	10.5 ± 0.3	16.6 ± 0.6	20.5 ± 0.6	114.3
	11.30:11.30	$25\pm1^{o}C$	10.5 ± 0.3	16.6 ± 0.5	22.5 ± 0.5	95.2
	Greenhouse Controls	$25\pm2^{o}C$	10.8 ± 0.5	15.0 ± 0.6	22.7 ± 0.6	110.1

Table 5.9 Effect of Temperature and Photoperiod on Leaf Production of Endurance Plants

	Photoperiod (light:dark, h, min)	Growing Temperature (^{o}C)	Leaf Production Day 0	Wk 6	Wk 12	Percentage Increase/Decrease in Leaf Numbers
	16:8	$10\pm1^{o}C$	11.0 ± 0.4	18.0 ± 1.4	24.0 ± 2.4	118.2
	16:8	$15\pm1^{o}C$	12.0 ± 1.0	16.0 ± 1.3	30.0 ± 2.4	150.0
	16:8	$25\pm1^{o}C$	13.0 ± 0.9	22.0 ± 1.3	31.0 ± 2.1	138.5
Optimal Cycle Lengths	16:16	$10\pm1^{o}C$	12.0 ± 0.6	15.0 ± 0.9	21.0 ± 1.9	75
	14.30:14.30	$15\pm1^{o}C$	13.0 ± 0.2	17.0 ± 1.4	23.0 ± 1.9	76.9
	11.30:11.30	$25\pm1^{o}C$	13.0 ± 0.8	18.0 ± 1.7	22.0 ± 1.8	69.2
	Greenhouse Controls	$25\pm2^{o}C$	13.0 ± 1.0	15.0 ± 1.2	20.0 ± 1.7	53.8

Table 5.10 Effect of Temperature and Photoperiod on Flower Stalk Production of Endurance Plants

	Photoperiod (light:dark, h, min)	Growing Temperature (^{o}C)	Flower Stalk Production Day 0	Wk 6	Wk 12
	16:8	$10\pm1^{o}C$	0	0.8 ± 0.3	3.5 ± 0.9
	16:8	$15\pm1^{o}C$	0	1.9 ± 0.4	6.6 ± 0.7
	16:8	$25\pm1^{o}C$	0	2.0 ± 0.4	3.6 ± 0.9
Optimal Cycle Lengths	16:16	$10\pm1^{o}C$	0	1.3 ± 0.4	3.2 ± 0.7
	14.30:14.30	$15\pm1^{o}C$	0	2.0 ± 0.5	4.6 ± 0.7
	11.30:11.30	$25\pm1^{o}C$	0	2.4 ± 0.5	3.4 ± 0.4
	Greenhouse Controls	$25\pm2^{o}C$	0	1.4 ± 0.3	3.5 ± 0.9

5.11 Effect of Temperature and Photoperiod on the Frequency of Chlorosis in Three African Violet Lines (%)

	Photoperiod (light:dark, h, min)	Growing Temperature (^{o}C)	Frequency of Plants and Leaves (in parenthesis) Showing Chlorotic Symptoms(%) Blue Rhapsody	Anther-Derived	Endurance
Optimal Cycle Length	16:8	$10\pm1^{o}C$	100($80.\pm10.5$)	100(67.4 ± 4.4)	25(5.8 ± 2)
	16:8	$15\pm1^{o}C$	100(25.3 ± 3.4)	0	0
	16:8	$25\pm1^{o}C$	0	0	0
	16:16	$10\pm1^{o}C$	100(100)	100(86.2 ± 5.2)	50(29.4 ± 4.6)
	14.30:14.30	$15\pm1^{o}C$	100(39.6 ± 1.6)	0	0
	11.30:11.30	$25\pm1^{o}C$	7.6(3.8 ± 1.1)	0	0
	Greenhouse Controls	$25\pm2^{o}C$	0	0	0

Table 5.12 Effect of Temperature and Photoperiod on Leaf/Shoot Growth of Three African Violet Lines

	Photoperiod (light:dark, h, min)	Growing Temperature (°C)	Leaf/Shoot Dry Weight (g) Blue Rhapsody	Anther-Derived	Endurance
	Day 0	-	0.52±0.02	0.54±0.04	0.58±0.08
Optimal Cycle Lengths	16:8	10±1°C	0.48±0.06(-8)	0.75±0.03(39)	2.25±0.27(288)
	16:8	15±1°C	1.1±0.05(112)	2.81±0.17(420)	4.03±0.30(595)
	16:8	25±1°C	1.28±0.08(146)	2.56±0.18(374)	2.64±0.21(355)
	16:16	10±1°C	0.44±0.04(-15)	0.68±0.02(25)	1.83±0.13(216)
	14.30:14.30:	15±1°C	1.08±0.06(108)	2.13±0.14(294)	3.32±0.21(472)
	11.30:11.30	25±1°C	1.29±0.09(148)	2.16±0.19(300)	2.48±0.21(328)
	Greenhouse Controls	25±2°C	1.22±0.06(134)	2.29±0.22(324)	2.57±0.27(343)

Percentage increase/decrease in dry weights compared to day 0 values shown in parenthesis

Table 5.13 Effect of Temperature and Photoperiod on Root Growth of thre African Violet Lines

	Photoperiod (light:dark, h, min)	Growing Temperature (^{o}C)	Dry Weight of Roots (g) Blue Rhapsody	Anther-Derived	Endurance
	Day 0	-	0.15 ± 0.01	0.13 ± 0.02	0.17 ± 0.7
Optimal Cycle Lengths	16:8	10 ± 1 C	0.09 ± 0.01(-40)	0.22 ± 0.04(69)	0.46 ± 0.09(170)
	16:8	$15\pm1^{o}C$	0.31 ± 0.2(106	0.56 ± 0.08(331)	1.35 ± 0.17(694)
	16:8	$25\pm1^{o}C$	0.49 ± 0.07(227)	0.53 ± 0.03(307)	0.59 ± 0.04(247)
	16:16	$10\pm1^{o}C$	0.04 ± 0.01(-73)	0.22 ± 0.05(69)	0.44 ± 0.06(158)
	14.30:14.30	$15\pm1^{o}C$	0.29 ± 0.04(93)	0.42 ± 0.06(223)	0.97 ± 0.22(470)
	11.30:11.30	$25\pm1^{o}C$	0.42 ± 0.05(180)	0.41 ± 0.09(215)	0.47 ± 0.44(176)
	Greenhouse Controls	$25\pm2^{o}C$	0.38 ± 0.03(153)	0.49 ± 0.1(277)	0.66 ± 0.05(65)

Percentage increase/decrease in dry weight compared to day 0 values shown in parenthesis.

Table 5.14 Effect of Temperature and Photoperiod on Flower Stalk Growth of Three African Violet Lines

	Photoperiod (light:dark, h, min)	Growing Temperature (^{o}C)	Dry Weight of Inflorescences (g) Blue Rhapsody	Anther-Derived	Endurance
	Day 0		0	0	0
	16:8	$10\pm1^{o}C$	0	0.02 ± 0.01	0.38 ± 0.08
	16:8	$15\pm1^{o}C$	0.0 ± 0.02	0.44 ± 0.1	0.63 ± 0.05
	16:8	$25\pm1^{o}C$	0.24 ± 0.03	0.44 ± 0.13	0.36 ± 0.1
Optimal Cycle Lengths	16:16	$10\pm1^{o}C$	0	0.01 ± 0.001	0.11 ± 0.02
	14.30:14.30	$15\pm1^{o}C$	0.13 ± 0.01	0.18 ± 0.02	0.47 ± 0.04
	11.30:11.30	$25\pm1^{o}C$	0.15 ± 0.05	0.21 ± 0.03	0.27 ± 0.07
	Greenhouse Controls	$25\pm2^{o}C$	0.17 ± 0.01	0.39 ± 0.05	0.31 ± 0.07

Flower stalk production was low in all the temperature and photoperiod regimes tested (Tables 5.4 and 5.14). Furthermore, plants grown under 25°C had flower stalks in an immature state. However, a trend was observed. Whereas plants grown at 15°C and 25°C produced flower stalks, plants grown at 10°C under either of the light regimes did not. Flower stalk production was significantly greater at 25°C under the 16h:8h compared to the optimum light:dark cycle.

Plants grown at 25°C under both the 16h:8h and optimal growth cycles performed similar with respect to leaf/shoot and flower production compared to plants grown under the brighter and hotter conditions of the greenhouse (Tables 5.12 and 5.14).

5.3.2 Anther-Derived Plants

Anther-derived plants Blue Rhapsody plants were tested for low temperature tolerance. After 12 weeks incubation at 10°C, chlorotic symptoms were observed in 67-87% of leaves of anther-derived plants grown under either the 16h:8h or the optimum 32h growth cycle (16h:16h) (Table 5.11). As with Blue Rhapsody plants (section 5.3.1) chlorosis was significantly higher on plants grown under the optimum compared to the 16h:8h, light:dark cycle (Tables 5.11 and Fig 5.2). Anther-derived lines grown under the 16h:8h, light:dark cycle exhibited more than a 38% increase in leaf/shoot biomass, after 12 weeks growth at 10°C (Table 5.12). However, plants grown at 10°C under the optimal growth cycle (16h:16h) showed a significant decrease in leaf/shoot biomass when compared with anther-derived lines grown under a 16h:8h, light:dark cycle (Table 5.12). Anther-derived lines incubated at 15°C under the optimal ligh/dark cycle showed a similar increase in leaf/shoot biomass compared to plants grown at


TEMPERATURE
10°C
15°C
25°C
16h:8h
Optimum
Blue Rhapsody
16h:8h
Optimum
Anther-Derived
16h:8h
Optimum
Endurance
PHOTOPERIOD (LIGHT:DARK)

FIG. 5·2

25°C in the growth cabinets, and glasshouse (Tables 5.5, 5.6 and 5.12). However plants grown at 15°and 25°C under the 16h:8h cycle always performed better, with respect to leaf/shoot biomass production, compared to plants grown at the same temperature under the optimum growth cycle lengths (Table 5.12)

Although plants grown at 10°C for 12 weeks showed a significant increase in root biomass, root production was greatest at 15°C and 25°C under the 16h:8h, light:dark cycle (Table 5.13).

After 12 weeks, production of flower stalks was observed at all the temperatures and light/dark cycles investigated (Tables 5.7i and 5.14). Compared to plants grown under glass and the 16h:8h cycle, the flower stalk production was always significantly less when plants were incubated under the extended light:dark cycles. Significantly, plants grown at 15°C under both the 16h:8h and optimal cycle lengths always performed similar to their respetive light:dark cycle counterparts incubated at 25°C. However, plants grown at 15°C and 25°C under the 16h:8h cycle produced two times more flower stalk biomass compared to plants grown at the same temperatures under the optimal cycle lengths (Table 5.14).

After 12 weeks greenhouse grown anther-derived plants showed a three fold increase in leaf/shoot biomass and a similar flower stalk production as plants grown at 15°C and 25°C under the 16h:8h, light:dark cycle.

5.33 Endurance Plants

Plants established from leaf cuttings of a cold tolerant variety of Endurance were evaluated. Chlorosis was observed in 5-29% of leaves of Endurance plants grown at 10±1°C (Table 5.11). Growing Endurance

plants at 10°C under the optimal growth cycle length (32h, 16h light:16h darkness) caused more than a five fold increase in the incidence of chlorosis (Blue Rhapsody, section 5.3.1 and Anther-Derived, section 5.3.2). However plants grown at 10±1°C under either the 16h:8h or optimal light/dark cycle length exhibited a 3.9 and 3.2 times increase in leaf/shoot biomass respectively (Table 5.12). Moreover, the highest growth rates were observed by plants grown at 15±1°C under both the 16h:8h, light:dark cycle (Tables 5.8, 5.9 and 5.12). Plants grown under the 16h:8h cycle at this temperature showed almost a seven fold increase in leaf/shoot dry weight after 12 weeks of growth (Table 5.12). Furthermore, it was observed that growing Endurance plants at 25°C caused a significant decrease in leaf/shoot biomass production compared to plants grown at 15°C under either the 16h:8h or optimal light:dark cycles (Table 5.12).

Similar to leaft/shoot biomass production root growth was highest at 15°C under the 16h:8h and 14h30min:14h30min, light:dark cycles (Table 5.13). The former treatment exhibited the greater increase in root dry weight than the latter. Plants grown at 10°C and 25°C always showed a significant decrease in root growth rates when compared to those grown at 15°C.

Flower stalk production was observed at all the temperatures and photoperiods investigated (Tables 5.10 and 5.14). Comparison of production, at each incubation temperature, between the 16h:8h and optimal growth cycle lengths (10°C, 32h; 15°C, 29h; 25°C, 23h) showed that plants grown at 15°C under the 16h:8h cycle always produced the most flower stalks (Table 4.12 and Fig 4.2). Furthermore, under both the 16h:8h and optimal growth cycles, plants grown at 15°C produced significantly more flower stalks

than those grown at 25°C (Tables 5.10 and 5.14).

After 12 weeks of growth, greenhouse grown plants showed a four fold increase in leaf/shoot biomass which was similar toplants grown in growth cabinets at 25°C undeer both the 16h:8h and optimal growth cycles at this temperature (Table 5.12). However flower stalk production under glass was low compared to plants grown at 15°C under the 16h:8h cycle (Table 5.14).

5.3.4 Evaluation of the Influence of Temperature and Photoperiods on Growth of Three African Violet Lines

Comparisons were made between growth data obtained from the three call lines of African violet namely Blue Rhapsody, anther-derived and Endurance.

5.3.4.1 Leaf/Shoot Production

At all temperatures and photoperiods investigated (including greenhouse grown controls) anther-derived plants always performed significantly better than the parent Blue Rhapsody line (Table 5.12). However, without exception the most productive line was Endurance plants grown at 15°C under the 16h:8h,dark:light cycle. Such plants exhibited almost a seven fold increase in leaf/shoot biomass after 12 weeks incubation (Fig 5.2 and Table 5.12). The poorest growth was recorded for Blue Rhapsody lines growen at 10 ± 1°C under the 16h, light:16h dark cycle (which showed a 15% decrease in leaf/shoot biomass.

16h:8h, Light:Dark Cycle Length

It was found that Blue Rhapsody always performed best at 25°C (Table 5.12). The growth performance of antehr-derived plants at 15°C is comparable to that obtained by the same line at 25°C (Table 5.12)

and significantly higher when compared to Blue Rhapsody plants grown at the same temperature (25°C). Endurance plants grown at 10°C perform significantly better than Blue Rhapsody plants grown at either 15° or 15°C (Table 5.12).

Optimal Growth Cycles

The effect of optimal cycle lengths on vegetative growth of plants differed for the three African violet lines investigated (Table 5.12). Whereas increase of Blue Rhapsody leaf/shoot biomass was independent of the optimal growth cycle lengths, at all three temperatures investigated, this was not the case in either the anther-derived or the Endurance lines. When compared to plants grown under the 16h:8h cycle, anther-derived and Endurance lines grown under the optimal cycle showed a significant decrease in production of leaf/shoot biomass at 10° and 15°C (Table 5.12). A similar significant decrease in leaf/shoot biomass (When comparing plants grown under the 16h:8h to the optimal cycle) wqs observed in anther-derived lines incubated at 25°C. Growth of Endurance lines at 25±1°C was independent of the optimum growth cycle length

Greenhouse Controls

The three lines of African violet grown at 25°C under both the 16h:8h and optimal light:dark cycles all performed comparably withtheir respective greenhouse controls (Table 5.12). However, whereas Blue Rhapsody plants grown at 15°C under either 16h:8h, light:dark cycle produced significantly less biomass compared to glasshouse grown African violets, anther-derived and Endurance lines produced significantly more biomass at the same temperature (Table 5.12). Furthermore Endurance lines grown at 15°C under the optimal growth cycle (14h30min

light: 14h30min darkness) had a significnatly higher growth rate compared to greehouse grown plants.

5.3.4.2 Root Production

Increase in root biomass exhibited a similar pattern to that observed for leaf/shoot growth (section 5.3.4.1, Table 5.12) by the three African violet lines. At 15°C under the 16h:8h, light:dark cycle anther-derived lines showed a signficant increase in root biomass compared to Blue Rhapsody plants. However, Endurance plants produced the highest root biomass, after 12 weeks incubation at 15°C (Table 5.13). Root growth was not significantly enchanced by growth of plants from the three lines at 10°, 15° and 25°C under either the optimal growth cycles (10°, 16h:16h; 15°, 14h30min:14h30min, 25°, 11h30 min, light:dark) or glasshouse conditions.

5.3.4.3 Flower Stalk Production

Using the data of increase in flower stalk dry weights as the most reliable measure of reproductive growth (Table 5.14) it was observed that Blue Rhapsody plants grown at 10°C under both the 16h:8h and optimal cycle lengths, did not produce any flower stalks after 12 weeks of incubation. At 10°C, 15°C and 25°C, the familiar pattern of growth is again observed. Anther-derived plants are always more productive thatn the Blue Rhapsody parent but the Endurance line produced the highest increase in flower stalk biomass after 12 weeks of growth. Whereas, Blue Rhapsody plants produced the most flower stalks at 25±1°C, anther-derived and Endurance lines performed equally as well at 15±1°C under the 16h:8h, light:dark cycle. Generally the growth of plants at the three temperatures under optimal cycle and greenhouse conditions caused a decrease in the production of flower stalk biomass.

Nevertheless, anther-derived lines grown at $15\pm1^{o}C$ and greenhouse grown Endurance plants displayed a slight but insignificant increase in biomass, when compared to their 16h:8h, light:dark controls.

5.3.5 Growth of the Three African Violet Lines at Low Temperature

Comparisons of plants uncubated at low temperatures, especially at $10^{o}C$ were made to investigate the ability of the three African violet lines to tolerate grow,and reproduce under such conditions.

All three African violet lines grown under the 16h:8h cycle displayed chlorotic symptoms at $10^{o}C$ (Table 5.11). However, chlorosis was also observed in Blue Rhapsody plants at $15^{o}C$ under both the 16h:8h and 16h:16h, light:dark regimes (Table 5.11). At $10^{o}C$, under the 16h:8h, dark:light cycle anther-derived lines displayed significantly less chlorotic symptoms than the Blue Rhapsody parent, but Endurance lines showed the least leaf chlorosis. Growth of the three plant lines under the optimal cycles at the above temperatures significantly increased the frequency of leaf chlorosis.

The data in Figs 5.3, 5.4 and 5.5 show that incubating Blue Rhapsody plants for 12 weeks at $10^{o}C$ under either the 16h:8h or the toptimal growth cycle was deterimental to vegetative and reproducive growth. However, anther-derived lines showed a net increase in leaf/shoot root and flower biomass after the same period of time. Comparison of the above line with a commercially available cold-tolerant variety (endurance) reveals that the tolerant plants grown under the same 16h:8h cycle produced about 3, 2 and 19 times more leaf/shoot, root and flower stalk tissues respectively.

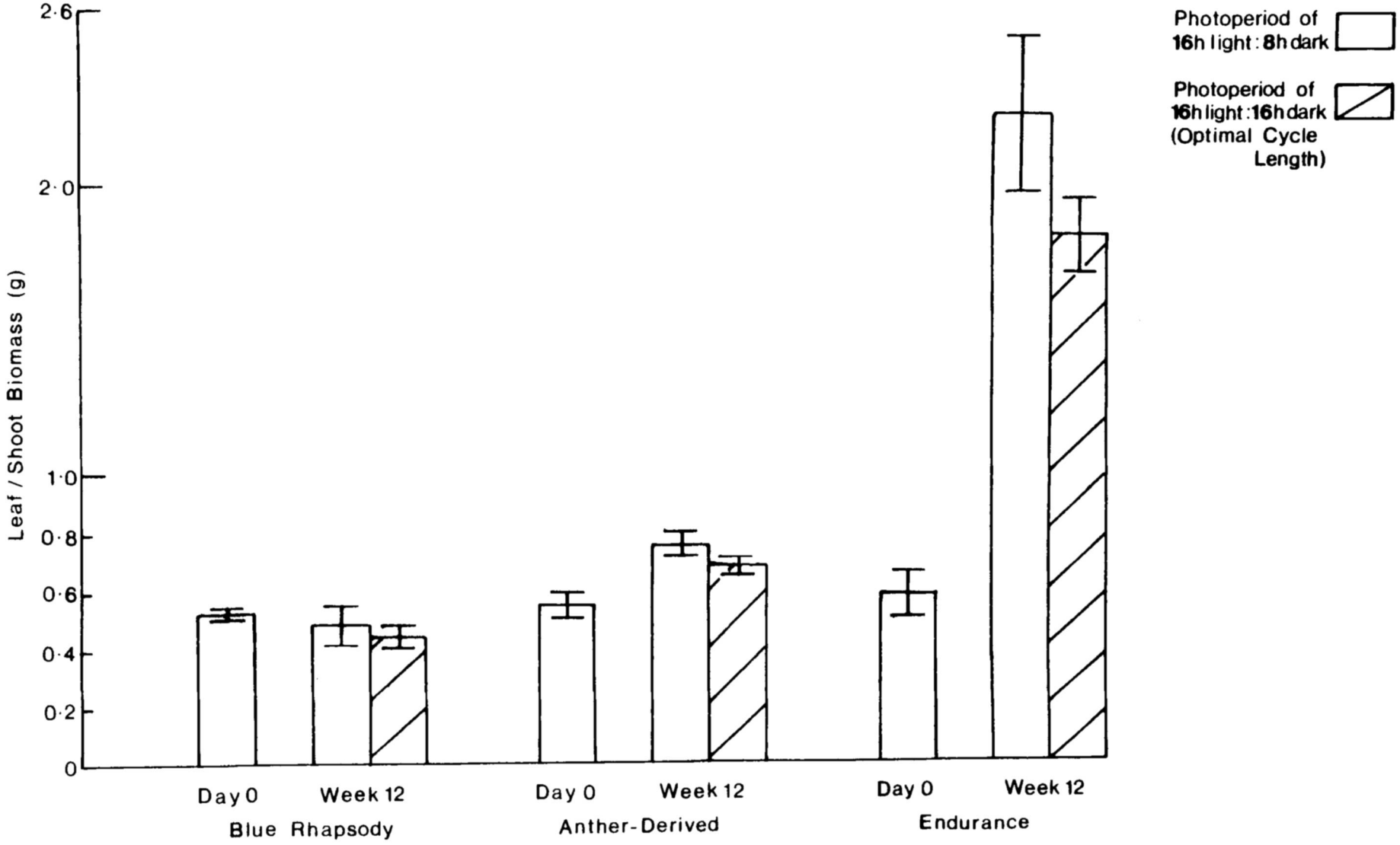


Fig. 5.3 Effect of Low Temperature (10oC) and Photoperiods on the Vegetative Growth of Three African Violet Lines.

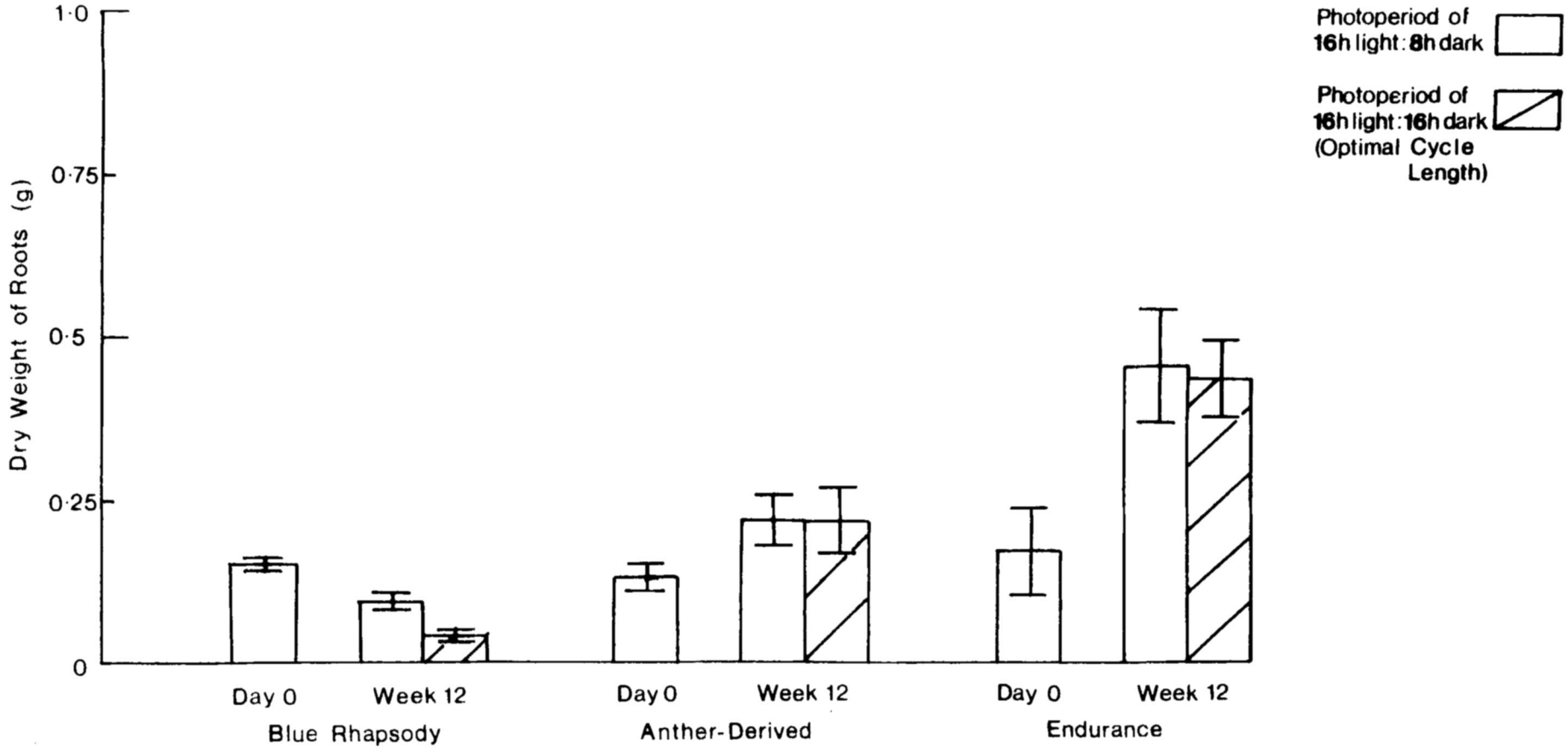


Fig. 5.4 Effect of Low Temperature ($10^{o}C$) and Photoperiods on Root Growth of Three African Violet Lines.

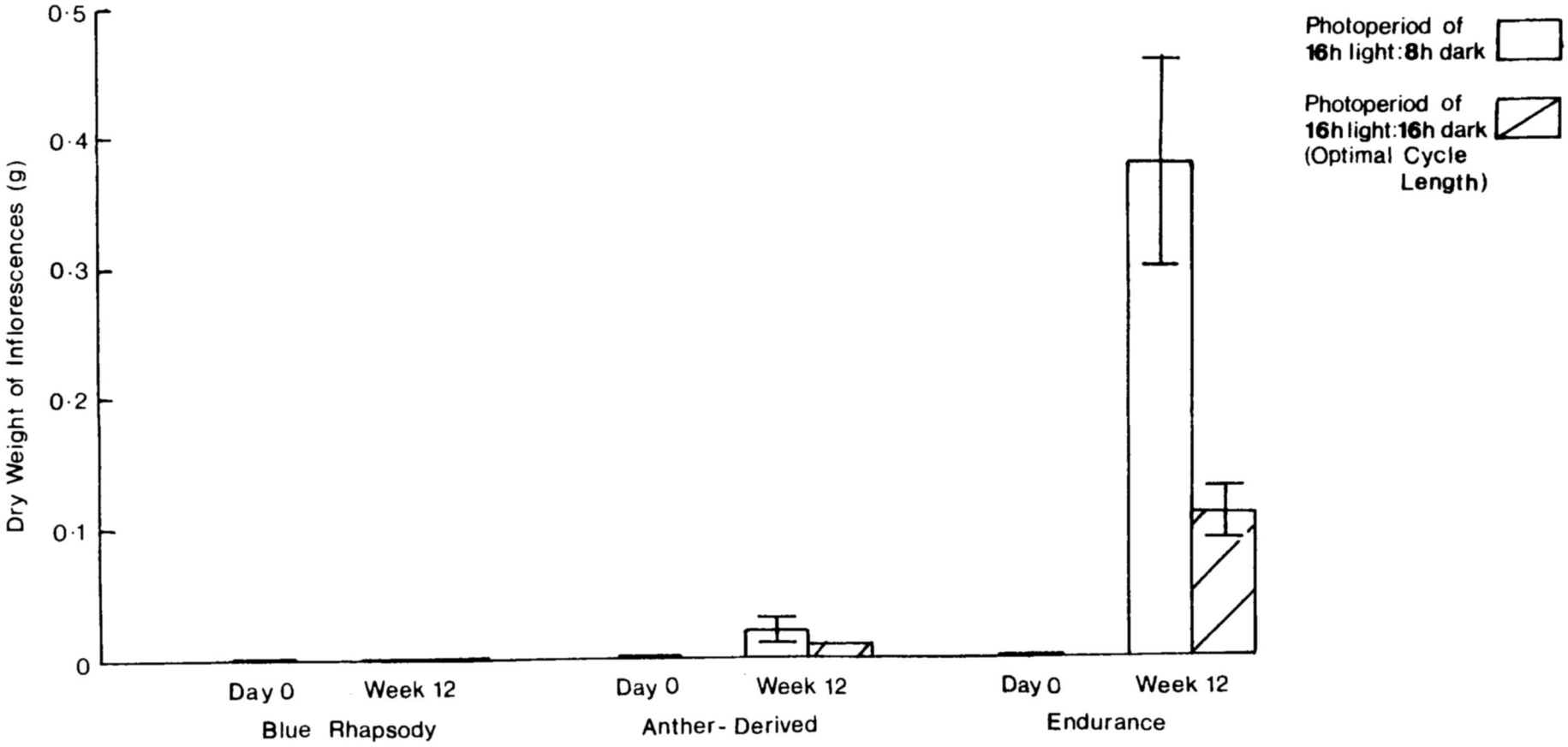


Fig. 5.5. Effect of Low Temperature ($10^{o}C$) and Photoperiods on Reproductive Growth of Three African Violet Lines

5.4 Discussion

Data from this study shows that $25\pm1^{o}C$ is the optimum growing temperature for growth development and flowering of the African violet cultivar Blue Rhapsody. This confirms the work of Went (1957), Herklotz (1968) and Hildrum and Kristoffersen (1969). These workers have shown that the optimum constant growing temperature for commercial lines African violets (grown under either natural or artificial light) was found to lie between $21-26^{o}C$.

Lowering the growing temperature under which Blue Rhapsody plants were grown resulted in a high frequency of leaf chlorosis and a significant decrease in leaf/shoot, root and flower stalks biomass. It was observed that Blue Rhapsody plants would not flower if grown at $10^{o}C$. Similar findings were reported by Went (1959) and Bilkey (1981). These workers found that the first symptoms of abnormal growth at low temperatures are a lack of chlorophyll formation causing yellowing of existing and newly formed leaves. Furthermore results from this investigation are in agreement with other authors who found that the minimum constant temperature which produces plants of marketable quality is $19^{o}C$ (Bilkey, 1981). Below $19^{o}C$ growth is impaired and at $15^{o}C$ and below flower development is inhibited in commercial Saintpaulias (Herklotz, 1968). However results in this study show that Blue Rhapsody plants derived from cultured anther-tissue can tolerate a lower growing temperature than the Blue Rhapsody parent. Moreover, anther-derived lines incubated at $15^{o}C$ exhibited a comparable increase in leaf/shoot, root and flower stalk dry weight to control plants grown at $25^{o}C$.

In 1981 Larkin and Scowcroft reported resistance to Downy mildew, Fiji and Eyespot disease by surgarcane somaclones regenerated from callus cultures.

However, to the authors knowledge no reports have been cited in the literature of increased low temperature tolerance in plants regenerated from anther callus. Therefore in conclusion, from this investigation, plants of marketable quality were obtained from anther-derived lines that tolerated incubation at 15°C.

The results in this study show that the most productive line was Endurance plants grown at 15°C under the 16h:8h; light:dark cycle. Incubating this line at temperatures above or below 15°C was inhibitory to plant growth and development. This work therefore confirms the cold tolerant and cold preferant nature of this line as observed by Bilkey (1981).

The data presented in this work shows that the growth of the three African violet lines at 10°, 15° and 25°C under the respective optimal cycle lengths (Went, 1959, 1960) (Figs 5.1 and 5.2) caused a significant increase in leaf chlorosis and a general decrease in leaf/shoot biomass of the anther-derived and Endurance but not the Blue Rhapsody plant lines. Furthermore, growth under the extended photoperiods generally caused a decrease in flower stalk production significantly so in anther-derived and Endurance lines . These observations are in direct contrast with the work of Went (1959, 1960) which shows that African violets incubated at 10°C and 15°C under a 32h and 27h or 30h cycle lengths respectively underwent normal development, were vigorous and flowered in abundance. Therefore to conclude it was notpossible to produce African violet plants of marketable quality by incubating them at lower temperatures under extended optimal photoperiods as described by Went (1959, 1960).

CHAPTER SIX
IN-VITRO SELECTION FOR COLD-TOLERANT, CELL LINES OF SAINTPAULIA IONANTHA

6.1 Introduction

In recent years the induction and selection of stress tolerant mutants or variants from plant cell and tissue cultures has received considerable attention (Handro, 1981; Tal, 1983). Isolated cells or protoplast cultures seem to be ideal systems for in-vitro selection since they avoid the problem of chimera formation. Furthermore, for the purpose of selection, such systems provide extremely large populations of individuals from which mutants or variants can effectively be screened under controlled chemical or physical stress conditions (Widholm, 1978). However, in many plant species, including Saintpaulia, the culture of single cells or isolated protoplasts is still problematical with regard to the induction of cell division and subsequent plant regeneration.

In leaf cuttings of Saintpaulia, plantlets are formed via adventitious shoots which generally arise from single epidermal cells (Broertjes, et al, 1968, 1978). Under appropriate conditions small organ fragments cultivated in-vitro retain this type of regeneration and at the same time show enhanced adventitious shoot formation. In comparison to isolated cell or protoplast cultures, the leaf culture system described is easy tohandle and regeneration occurs readily. Both conventional cuttings (Warfield, 1973; Broertjes, et al, 1978) and in-vitro leaf tissue explants (Grunewaldt, 1983; Geier, 1983) have been used in mutagenesis studies.

Due to increasing costs of glasshouse heating, tolerance to low temperatures has become a primary goal in breeding in several ornamentals. Probably this goal can be attained faster with less space being required by the use of in-vitro rather than

conventional selection methods (Walther and Preil, 1981, Preil, et al, 1983). Work by Schlegel (1982) found that African violet explants cultured in-vitro for 16 weeks showed no regeneration at temperatures below 15°C, although a few clones survived at 12°C. Recently Geier (1983) found that untreated or mutagen treated leaf segments, from five different Saintpaulia cultivars, incubated at 16°C for 9 months produced a limited number of adventitious shoots. However, these shoots were not regenerated into plants that could be screened at low temperatures for cold-tolerance.

6.2 Aims and Experimentation

The aim of this investigation was to test for low temperature tolerance of three African violet lines. Low temperature tolerance was determined by measurement of electrolyte leakage from excised leaf-discs. Leaf-discs were isolated from mature leaves of Blue Rhapsody diploid and anther-derived lines and Endurance plants then incubated on deionized water at 5°, 10°, and 25°C. At least five discs were used per treatment and each treatment was replicated 12 times. Furthermore the whole experiment wss repeated three times and the results were combined before analysis (Chapter 2, section 2.14).

In order to meet the above aim it was necessary to devise an appropriate in-vitro system. This necessitated the determination of the minimum temperature at which the three African violet lines could be grown. For each treatment, fifty 1cm leaf-discs were excised from mature leaves and plated, adaxial surface down, on solidified MS medium supplemented with NAA:BAP, 0.5:0.5mgl^{-1}. Cultures were incubated for 8 weeks at 5°, 10°, 15°, 20° and 25°C to determine the frequency of survival and growth of leaf-discs at each temperature. As before this experiment was duplicated and results were pooled prior to analysis.

In order to increase the success of selection for tolerance a mutagenesis step was employed. Therefore, the appropriate concentration at which the mutagen EMS could be applied to leaf-discs was determined (LD_{50}). Anther-derived surface sterilised leaf-discs were exposed to 1, 2,3, 4, 5 and 10% levels of EMS (½h) made up in 0.01M Phosphate buffer. Discs were cultured as above, incubated at 25°C and assessed for viability (percentage area of leaf tissue still green and not necrotic after 24h, 48h, 72h and 14d). Fifty leaf- discs were used for each treatment. The experiment was repeated and the results were combined prior to analysis.

This study also compared the viability of un-treated (control) and mutagen treated leaf-discs from the three African violet lines following incubation for 6 months at 10°C. For each treatment fifty leaf-discs were excised and cultured on MS medium supplemented with NAA:BAP, 0.5:0.5mgl^{-1} then incubated at 10°C. After six months, viability and adventious shoot production were measured for all leaf-discs. Explant biomas was determined for a sample of 25 leaf discs from each treatment. The experiment was repeated and results were pooled prior to analysis.

Following selection for tolerance in-vitro, surviving lines were used for the regeneration of plantlets. Therefore, after six months incubation at 10°C leaf-disc culture or surviving portions were selected and transferred to 25°C. After six weeks, regeneration was measured by increase in explant area. Thereafter adventitious shoots were selected from the regenerated explants and transferred to MS basal medium and incubated in the light (16h day length) at 25°C. After 8 weeks growth plantlets were transferred to compost and grown for 2 months in the greenhouse.

The final test in this selection procedure was to determine whether the higher tolerance to cold of the anther-derived and Endurance lines compared to the Blue Rhapsody diploid line, observed in the previous chapter (5.3) was retained. Ten to fifteen regenerated plants from untreated or mutagen treated leaf-discs of the three lines under investigation were incubated at $10\pm1^{o}C$. After 12 weeks growth was evaluated. The following parameters were measured: plant diameter, leaf number, leaf/shoot biomass, flower stalk number and biomass and the frequency of leaf chlorosis.

6.3 Results

6.3.1 Electrolyte Leakage

The analysis of electrolyte leakage (Chapter 2, section 2.14) from isolated leaf-discs incubated at $25^{o}C$ (Fig 6.1) revealed that leakage was very similar for the three lines under investigation. At $10^{o}C$ leakage from Blue Rhapsody leaf-discs was significantly higher when compared to those incubated at $25^{o}C$. However, no significant change in electrolyte leakage was recorded for the other two lines at $10^{o}C$. At this temperature both anther-derived and Endurance lost 15% less electrolytes than Blue Rhapsody indicating that these lines were more tolerant in this respect. However, incubating leaf-discs at the lower temperature of $5^{o}C$ caused a significant increase of electrolyte leakage from all three lines when compared to discs incubated at $25^{o}C$ (Fig 6.1). Thus indicating a lack of tolerance by Blue Rhapsody (diploid and anther-derived lines) and Endurance to this low temperature.

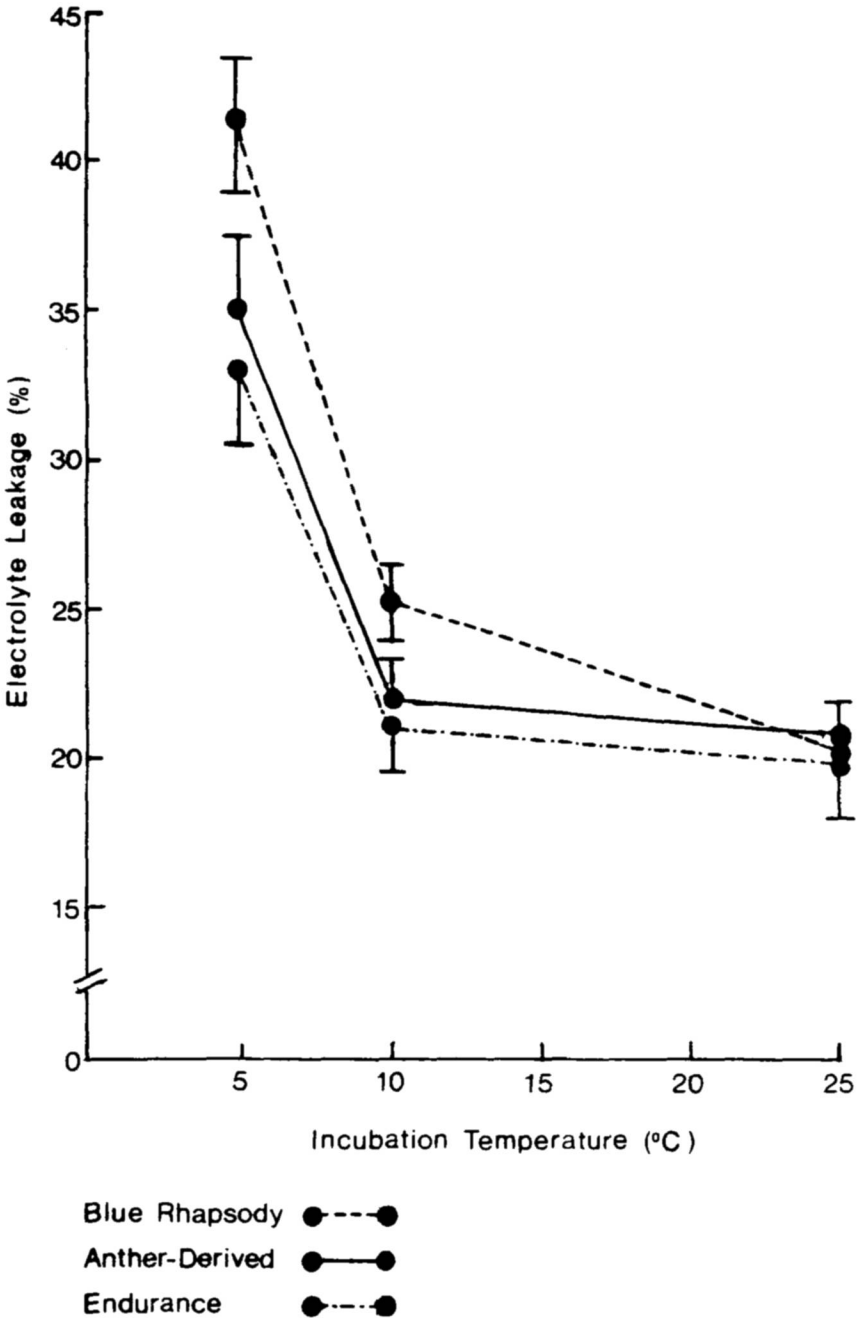


Fig. 6.1 The Effect of Temperature on the Rate of Electrolyte Leakage.

For each treatment five,1 cm leaf-discs were isolated from mature leves of three African violet lines and incubated on deionized water inthe dark at 5^{o}, 10^{o} and 25^{o}C. After 24h conductivity readings were taken. Thereafter percentage electrolyte leakage was determined. Each treatment was replicated 36 times. Standard errors are indicated.

6.3.2 Effect of Temperature on Survival and Growth of In-Vitro Cultured Leaf-Discs

In a preliminary comparative study, to determine the low-temperature tolerance of African violet tissue, leaf discs were excised from three Saintpaulia lines (Blue Rhapsody diploid and anther-derived plants and the cold-tolerant variety Endurance), cultured on MS medium supplemented with NAA:BAP, 0.5:0.5mgl^{-1} and incubated at 5°, 10°, 15°, 20° and 25°C.

Noe of the leaf-discs from the three AFrican violet lines incubated at 5°C survived (Fig 6.2). After 8 weeks of culture the whole disc tissue was brown and necrotic with no observable portion of the leaf-disc surviving. However, 7-29% of leaf-disc explants incubated at 10°C survived. Data in Fig 6.2 shows that the survival rate of the anther-derived leaf-disc explants is more than twice that of the diploid Blue Rhapsody parental leaf tissue. Furthermore, the growth rate of anther-derived itssue is more than 1.5 times that of the diploid parent (Fig 6.3). Comparison of the above cell lines with those derived from the commercially available cold-tolerant African violet (var Endurance) revealed that Endurance had the highest frequency of survival and that both Endurance and anther-derived leaf-disc explants produced between 40-60% more tissue than the Blue Rhapsody parent (Fig 6.3). Incubating leaf-discs at 15°C increased the survival rate of the disc tissue and consequently the amount of biomass they produced.

At 15°C the survival rate of Blue Rhapsody more than tripled, when compared to 10°C values, and one out of every four explants tolerated

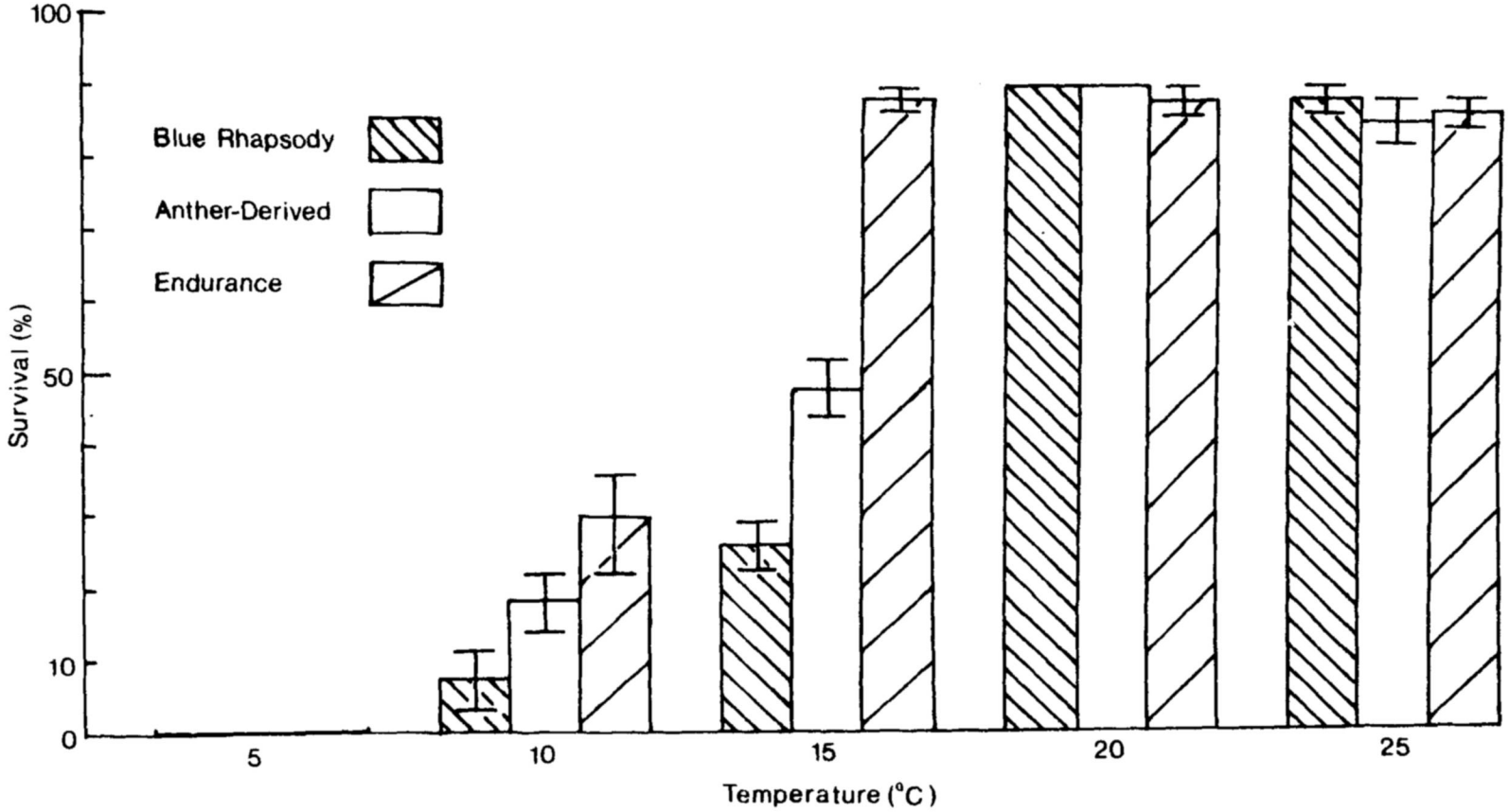


Fig. 6.2 Survival of Three Saintpaulia Lines Incubated at Various Temperatures for Eight Weeks. One cm leaf-discs from the three Saintpaulia lines under investigation were cultured. (adaxial surface down) on MS medium supplemented with NAA:BAP, 0.5: 0.5mgl^{-1} and incubated at 5^{o}, 10^{o}, 15^{o}, 20^{o} and 25^{o}C. Photoperiod was 16h light per day. After 8 weeks the frequency of surviving (non-necrotic and callusing) leaf-discs was determined. Fifty replciates were used for each treatment. The experiment was duplicated. Standard errors are indicated.

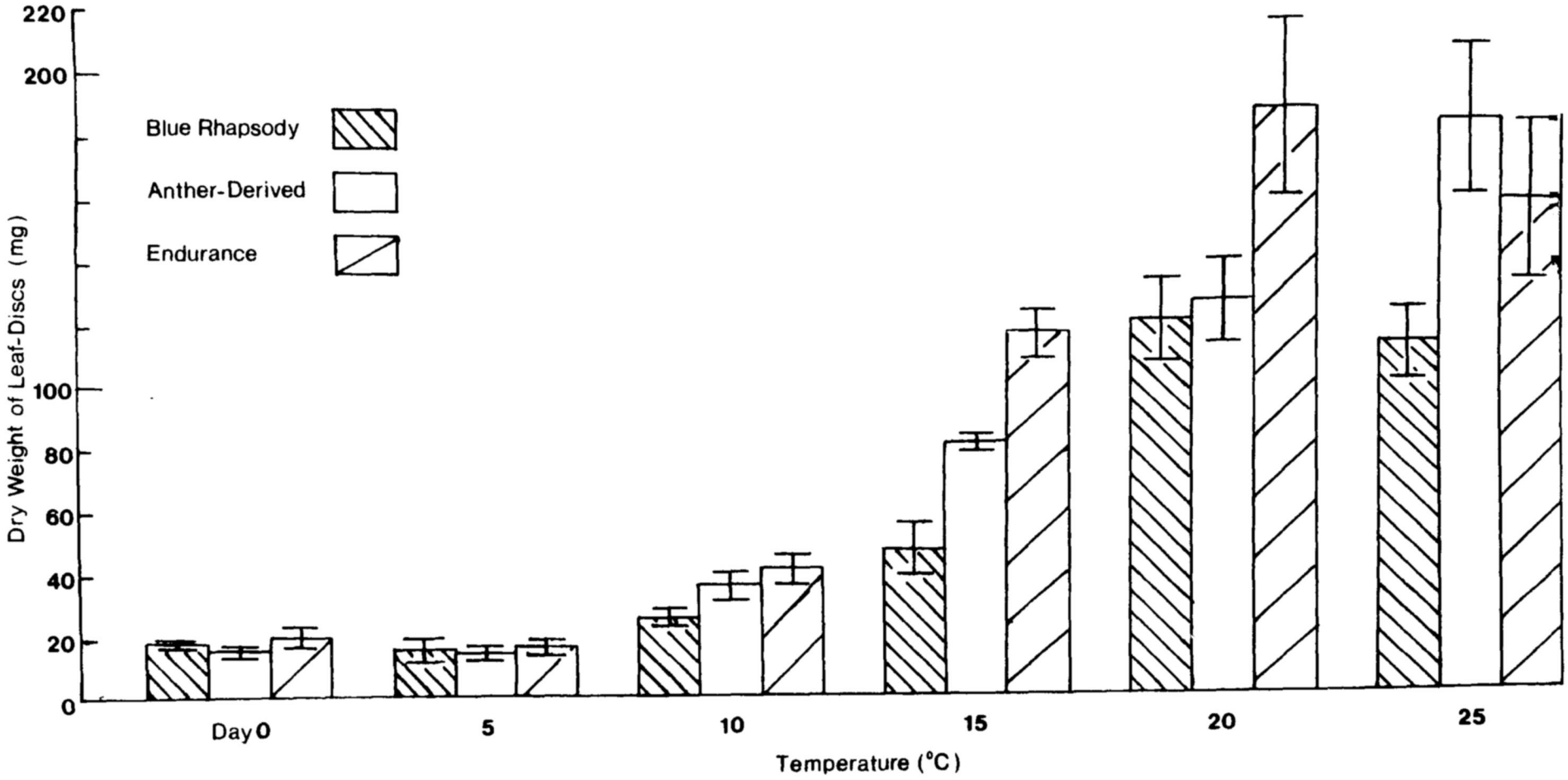


Fig 6.3 Biomass of Three Saintpaulia Lines Incubated at Various Temperatures for Eight Weeks. One cm leaf-discs from thre three Saintpualia lines under investigation were cultured adaxial surface down on MS medium supplemented with NAA:BAP; 0.5:0.5mgl^{-1} and incubated at 5^{o}, 10^{o},15^{o},20^{o} and 25^{o}C. Photoperiod was 16h light per day. After 8 wk dry weights were determined. Fifty replicates were used for each treatment. The experiment was duplicated and standard errors are indicated.

being subjected to low temperature stress (Fig 6.2). Significnatly, almost twice as many alnther-derived explants survived compared to the diploid parent. However,88% of Endurance leaf-discs survived 8 weeks incubation at 15°C. Compared to explants grown at 10°C, leaf-discs cultured at 15°C produced between 1.8 (Blue Rhapsody parent) -2.8 (Endurnace) more biomass. Moreover, at 15°C both anther-derived and Endurance explants produced respectively 180% and 250% more tissue than the Blue Rhapsody parent.

Almost all leaf-disc explants cultured at 20°C survived. Compared to cultures grown at 15°C, all three Saintpaulia lines showed a marked increase in growth rate. Whereas, the diploid and anther-derived lines of Blue Rhapsody had a similar growth rate. Endurance explants grew faster producing 150% more itssue (Fig 6.3). At 20°C both Endurance and the Blue Rhapsody parent lines showed optimal growth.

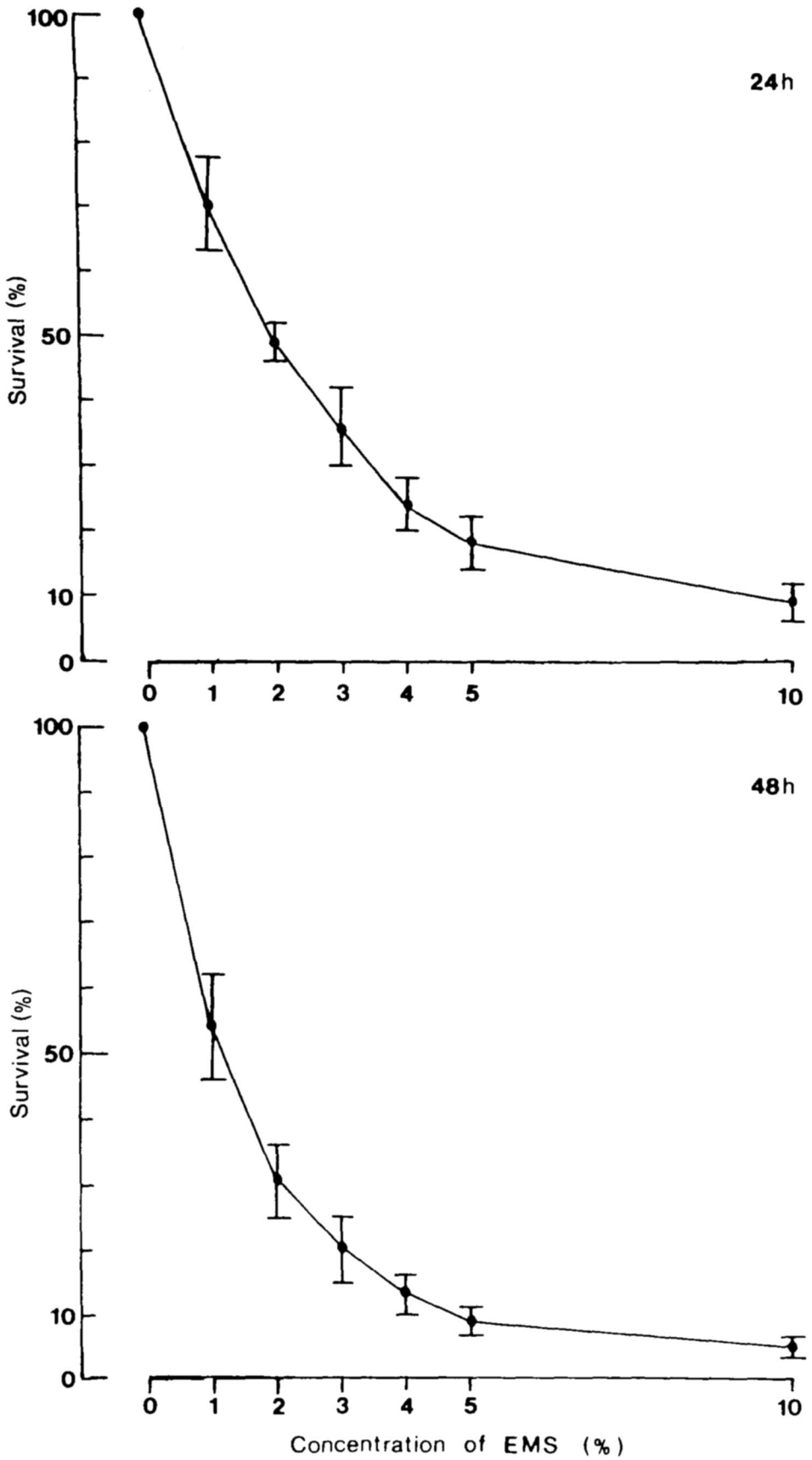
Culturing Saintpaulia leaf-discs at 25°C caused a slight decrease in their rate of survival (Fig 6.2). However the majority of discs (90-100%) survived. Compared to explants grown at 20°C the Blue Rhapsody diploid parent and Endurance lines exhibited a 7% and 16% mean decrease in explant dry weight. However, at 25°C the Blue Rhapsody anther-derived line showed a 45% increase in biomass compared to discs grown at 20°C.

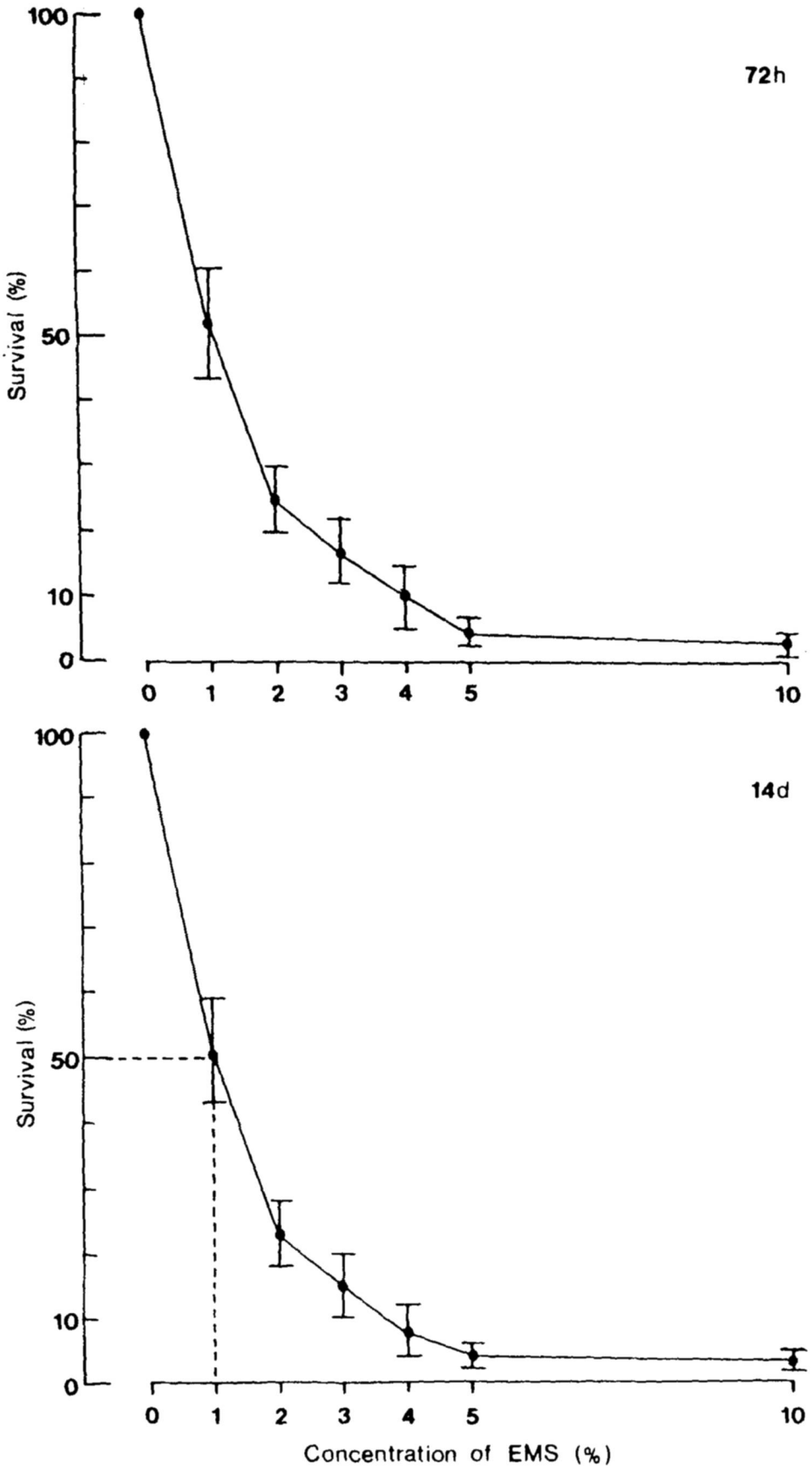
6.3.3 Effect of EMS on the Survival of Leaf-Disc Tissue From Anther-Derived Blue Rhapsody Plants

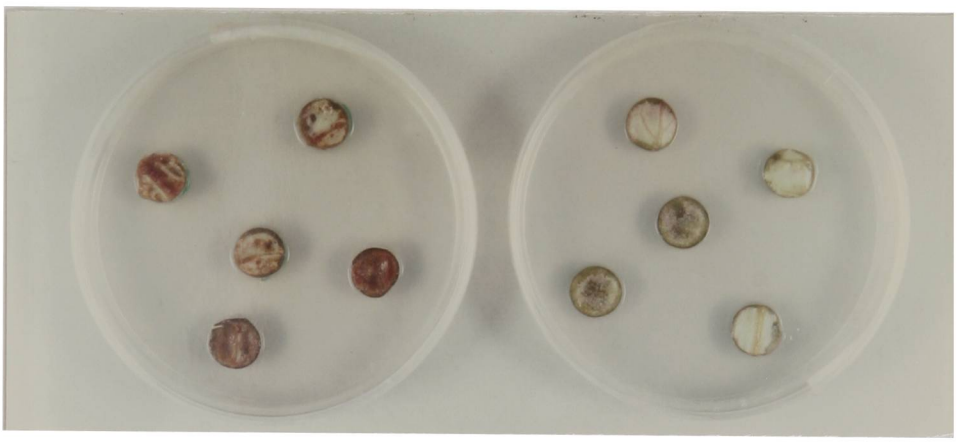
Excised leaf-discs from anther-derived Blue Rhapsody plants were exposed to various concentrations of EMS in 0.01M phospate buffer for ½ h to determine the LD50 (the concentration of EMS that will cause necrosis in 50% of a leaf-disc). After the mutagen was applied, leaf-discs were plated on MS medium supplemented with NAA:BAP (0.5: 0.5 mgl^{-1}) and incubated at 25°C (Fig 6.5).

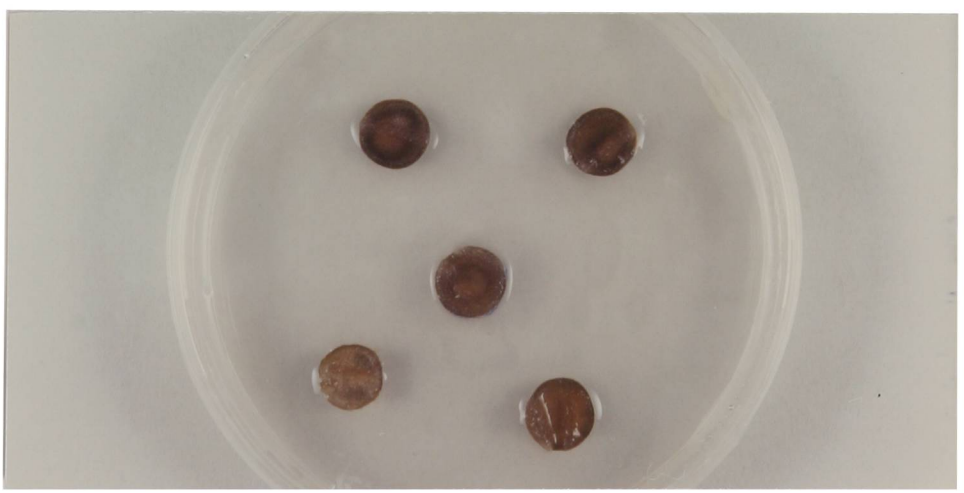
When only phosphate buffer was applied, it was observed that after 24h exposure that 100% of the leaf-disc surface remained, green, non-nectrotic and therefore viable (Fig 6.4). Application of 1% EMS to anther-derived leaf-disc caused a 30% loss of viable tissue (Fig 6.5a). Fifty per cent of leaf-disc tissue became necrotic when EMS was applied at a concentration of 2%. Increasing the concentration of EMS above 2% increased the damage to the leaf-disc tissue. The most extreme treatemnt, 10% EMS (½h) left only 10% of the disc tissue undamaged.

However, leaf-disc viability also decreased with time. Thus after 48h of culture only 30% of a leaf-disc was ot necrotic after being treated with 2% EMS and this is a drop of 20% of that found after 24h exposure. Furthermore, the leaf-discs treated with 1% had now an average viability of 54% Fig 6.4). After 72h the rate of decline of leaf-disc tissue viability had decreased (Fig 6.4) and by 14d had practically ceased. Whereas after 14d dosages between 5-10% EMS left a very low percentage area of leaf-disc tissue undamaged (Figs 6.4 and 6.5c), a dosage of 1% for ½h intact.


24h
48h
Survival (%)
100
50
10
0
0
1
2
3
4
5
10
Concentration of EMS (%)


72h
14d
Survival (%)
100
50
10
0
0
1
2
3
4
5
10
Concentration of EMS (%)





Therefore this dosage was used for all future mutagenic treatments.

6.3.4 Effect of Low Temperature on the Growth and Development of Cultured Leaf-Discs

Leaf-disc explants of three Saintpaulia lines were either left untreated (control) or treated with 1% EMS for ½h piror to incubation at 10°C for six months. Comparative studies were made between control and mutagen treated leaf-discs to evaluate the survival of leaf-disc material (measured as the percentage area of leaf-disc tissue still green and not necrotic). This was determined after 6, 12 and 24 weeks (Figs 6.6, 6.7 and 6.8). Leaf biomass (Fig 6.9) and the number of shoots produced per explant were measured after six months of incubation at 10°C (Figs 6.10 and 6.12).

6.3.4.1 Survival of the Leaf-Disc Cultures

After six weeks of culture at 10°C it was observed that between 44-69% of untreated leaf-disc tissue survived. This was determined on the basis of the percentage area of each leaf-disc tissue that was still green and not necrotic. Data in Fig 6.6 shows that on average 48% of leaf-disc tissue from Blue Rhapsody plants (diploid line) remained undamaged. Anther-derived leaf-disc tissue displayed a similar frequency of survival when compared to the Blue Rhapsody diploid line. However, after six weeks culture at 10°C Endurance leaf-disc tissue showe the highest frequency of survival (Fig 6.6). On average greater than 60% of each eaf-disc remained green and not necrotic. Furthermore, Endurance discs survived significnatly better than the Blue Rhapsody parent but not the anther-derived lines (Fig 6.6). Whereas the frequency

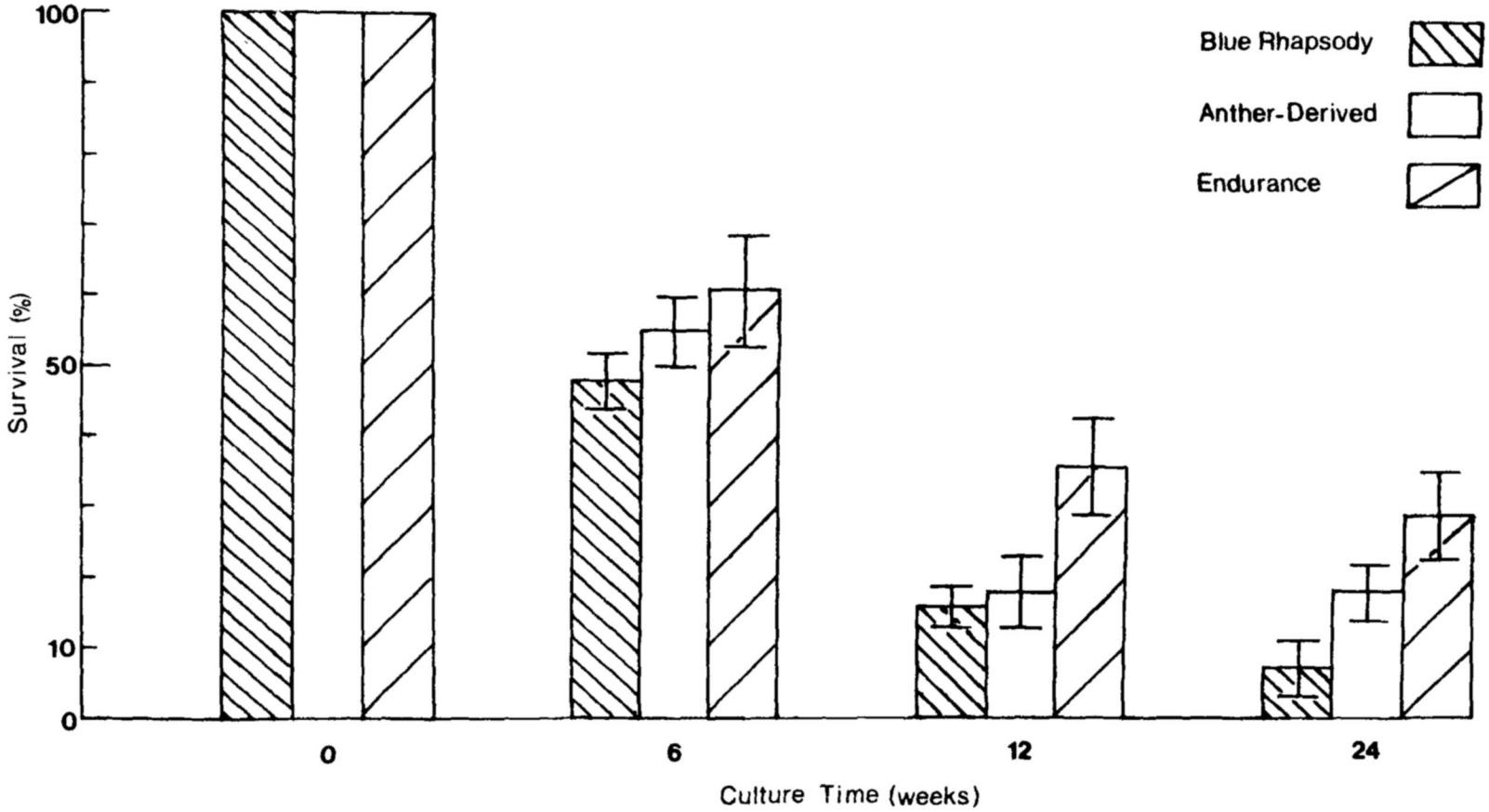


Fig 6.6 The Effect of Low Temperature ($10^{o}C$) on the Survival of Cultured Leaf-Disc Tissues. One cm leaf-disc from the three Saintpualia lines under investigation were cultured, adaxial surface down, on MS medium supplemented with NAA:BAP; $0.5:0.5mgl^{-1}$ and incubated at $10^{o}C$ with a 16h day length for 24 wks. Survival was measured as the percentage area of leaf-disc tissue that was non-necrotic and green . Fifty leaf-discs were used for each treatment. The experiment was duplicated. Standard errors are indicated.

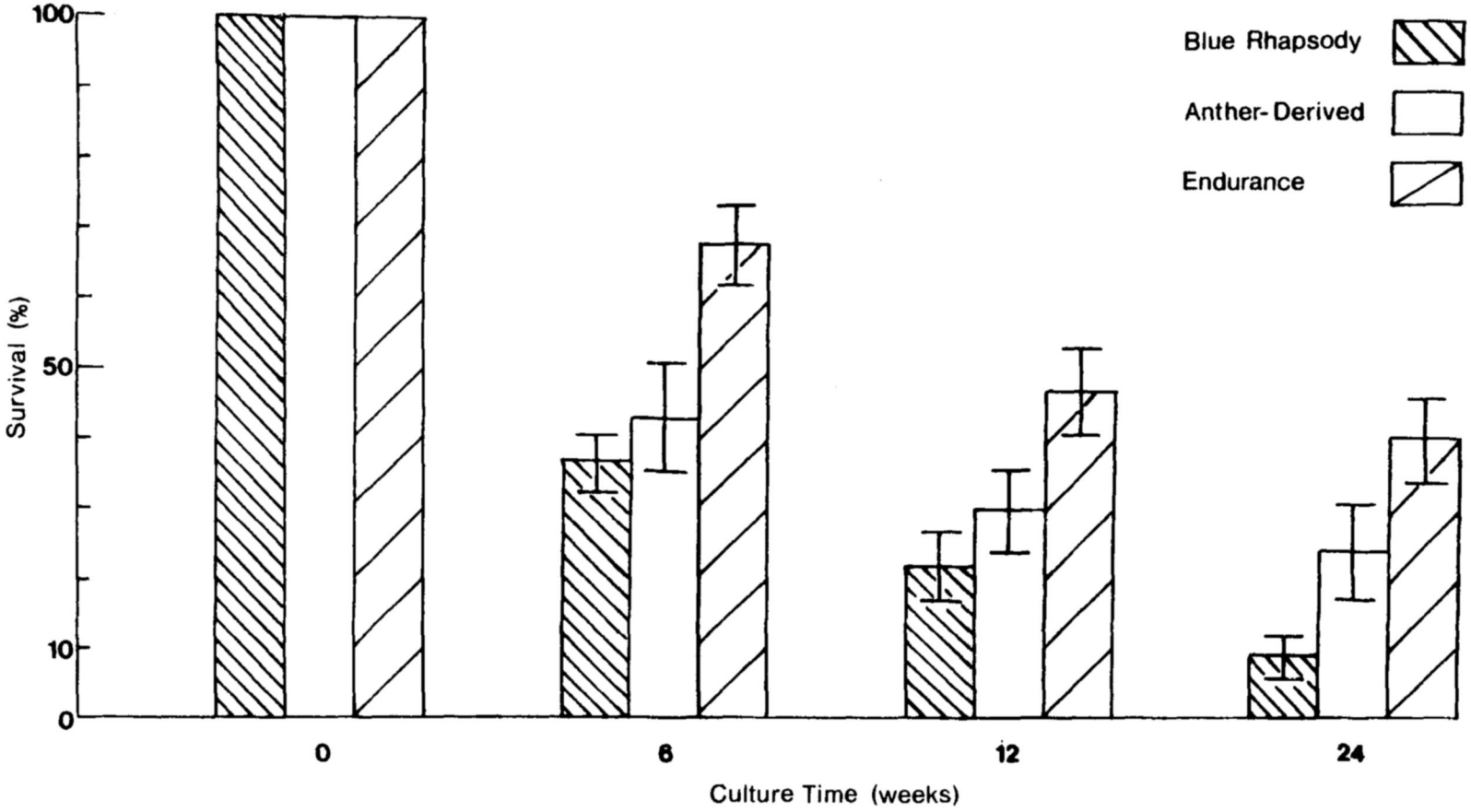


Fig 6.7 The Effect of the Mutagen EMS (1%, ½h) and Low Temperature (10^{o}C) on the Survival of Cultured Leaf-Disc Tissues. One cm leaf-discs from the three Saintapulia lines under investigation were exposed to a 1% concentration of EMS (made up in 0.01M phosphate buffer, pH7) for ½h, then incubated for 24wks at 10^{o}C with a 16h day length on MS medium supplemented with NAA:BAP, 0.5:0.5mgl^{-1}. Survival was measured as the percentage of area of leaf-disc tissue that was non-necrotic and green. Fifty leaf-discs were used for each treatment. The experiment was duplicated. Standard errors are indicated.

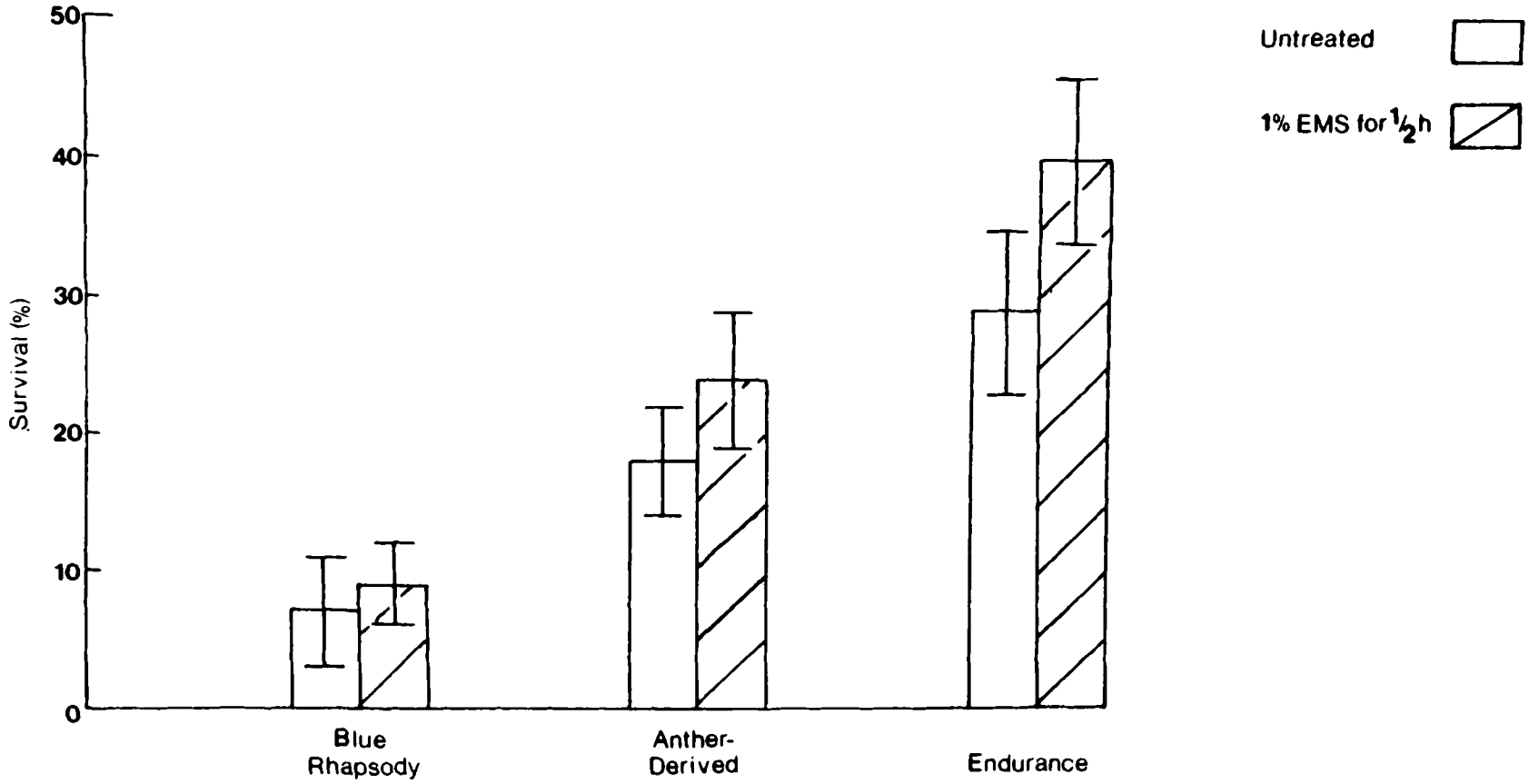


Fig 6.8 Survival of Leaf-Disc Tissue of Three Saintpaulia Lines Incubated for 24 WEeks. Untreated (control0 or EMS (1%, ½ hr) treated leaf-discs from three Saintpaulia lines were incubated for 24 wk at 10°C with a 16h day length, on MS medium supplemented with NAA:BAP, 0.5:0.5 mgl^{-1} Survival was measured as the percentage of leaf-disc tissue that was not necrotic and green. Fifty leaf-discs were used for each treatment The experiment was duplicated. Standard errors are indicated.

6.3.4.2 Growth and Development of Cultured Leaf-Discs Incubated at 10°C

Data represented in Fig 6.9 shows that the growth rate of untreated leaf-discs excised from anther-derived Blue Rhapsody plants is more than twice that of the diploid Blue Rhapsody leaf-discs. Furthermore, anther-derived tissue more readily underwent morphogenesis and produced about 30% more shoots than the diploid line (Fig 6.10). After four months of culture adventitious shoot formation started close to the cut edges of the explant (Fig 6.11) and by six months they developed over the entire surface of the leaf discs (Fig 6.13a). Often the developing shoots were partially chlorotic (Fig 6.13a). Comparison of the above cell lines with those derived from the commercially available cold-tolerant variety (Endurance) showed that both Endurance and anther-derived leaf-disc explants produced about 38-50% more tissue than the Blue Rhapsody parent (Fig 6.9). Furthermore, comparison of the morphogenetic potential of the three call lines, when cultured at 10°C, indicated that Endurance produced about 200% more shoots than the Blue Rhapsody parent but only 35% more than the anther-derived line (Fig 6.10).

Leaf-discs treated with 1% EMS prior to incubation at 10°C for 6 months, showed a similar increase in biomass when compared to the control explants from the same Saintpaulia lines (Fig 6.9). Also, a higher morphogenetic potential was observed in the leaf-disc explants treated with EMS. Blue Rhapsody (parent), anther-derived and Endurance leaf-discs produced respectively 7%, 32% and 31% more shoots when compared to control material. However such increases in shoot production were not statistically significant.

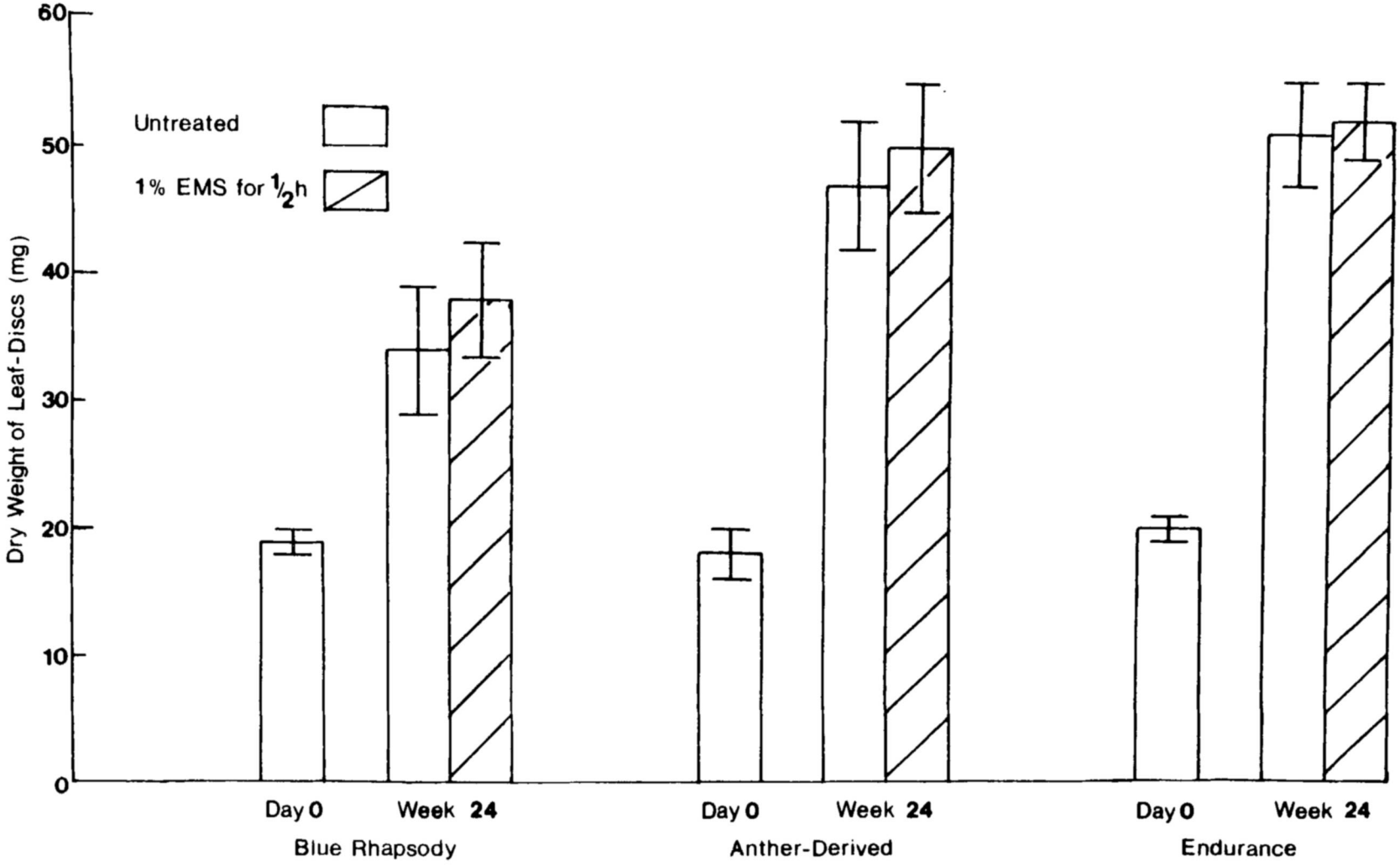


Fig. 6.9 Growth of Saintpualia Leaf-Disc Cultures at 10°C. Untreated or EMS (1% , ½h) treated leaf-discs from three Saintpaulia lines were incubated for 24 wk at 10°C with a 16h day length on MS medium supplemented with NAA:BAP,0.5:0.5mgl-1. Fifty leaf-discs were used for each treatment. The experiment was duplicated. To determine explant biomass 25 leaf-discs were samples from each treatment. Standard errors are indicated.

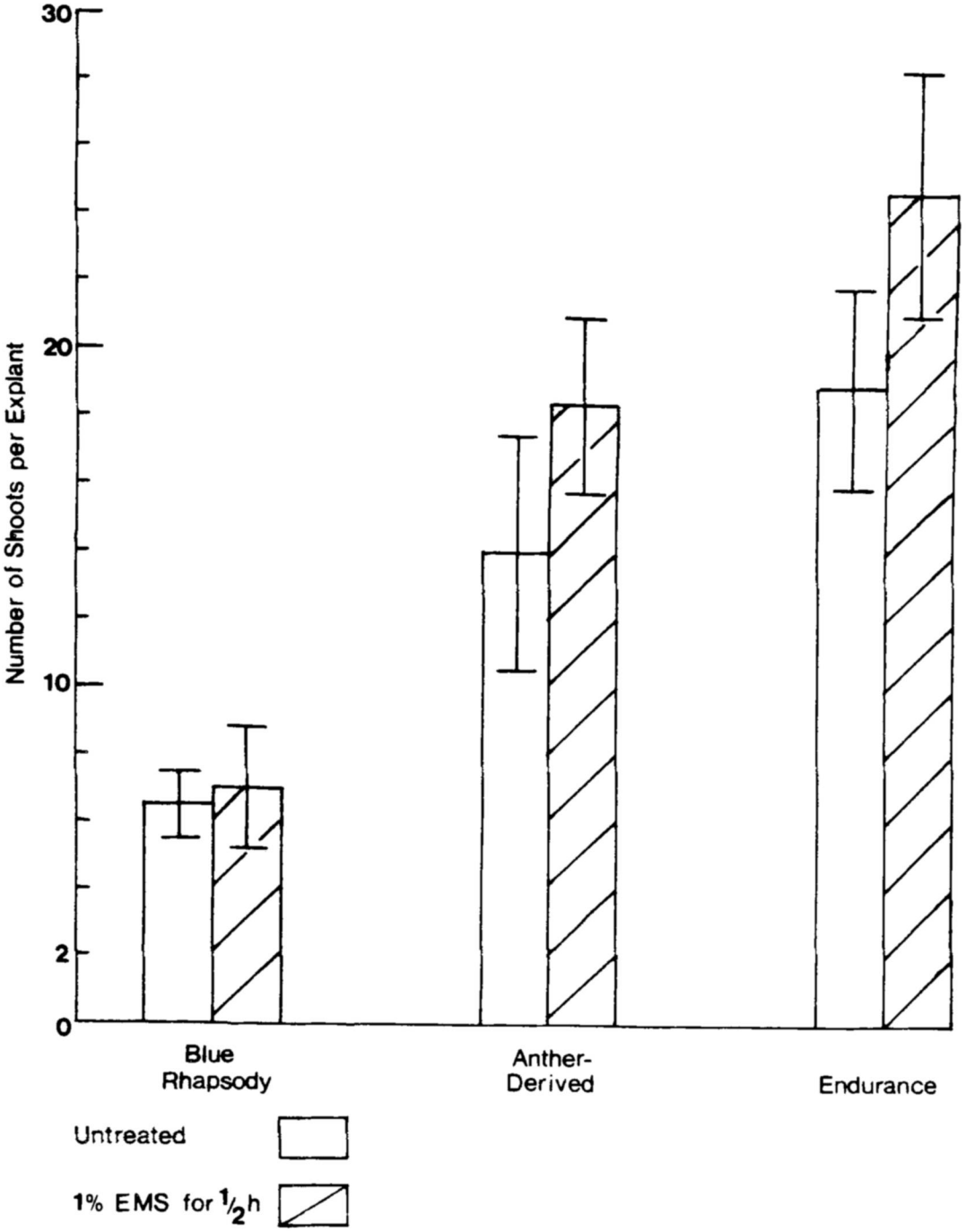


Fig 6.10 Adventitious Shoot Production from Leaf-Discs Cultured at 10°C. Untreated or EMS treated (1%, ½h) leaf-discs from three Saintpaulia lines were incubated for 24 wk at 10°C with a 16h day length on MS Medium supplemented with NAA:BAP; 0.5:0.5mgl^{-1}. Fifty leaf-discs were used for each experiment. The experiment was duplicated. Standard errors are indicated.

of green and non-necrotic leaf-disc tissue decreased to below 20% for both Blue rhapsody lines, a significantly higher frequency (36%) of leaf-disc surface still remained undamaged after 12 weeks of culture (Fig 6.6).

After 24 weeks incubation at 10°C only 18½ of the anther-derived leaf-disc tissue survived. However, this value was more than twice that of the Blue Rhapsody parent, but less than two thirds of the survival frequency obtained for Endurance tissue cultured for the same length of time (Fig 6.6).

For the three African violet lines tested a similar pattern of decline in leaf-disc tissue survival was observed in tissue treated with 1% of the mutagen EMS (Fig 6.7). Furthermore, leaf-disc tissue treated with mutagen did not show a significantly higher rate of survival than untreated tissue after 12 and 24 weeks incubation at 10°C (Figs 6.7 and 6.8). Nonetheless what is significant from this series of experiments is that on average between 7 and 29% of uhtreated leaf-disc tissue was not necrotic after 24 weeks incubation at 10°C. Furthermore, the anther-derived line has significantly 11% less necrotic tissue per leaf-disc than the Blue Rhapsody anther-derived line. However, Endurance leaf-discs have the lowest frequency of necrosis recorded (71%) (Fig 6.8). Pretreatment of African-violet leaf-disc tissue with 1% EMS for ½h prior to incubation at 10°C does not significantly decrease the frequency of necrosis per disc (Fig 6.8).

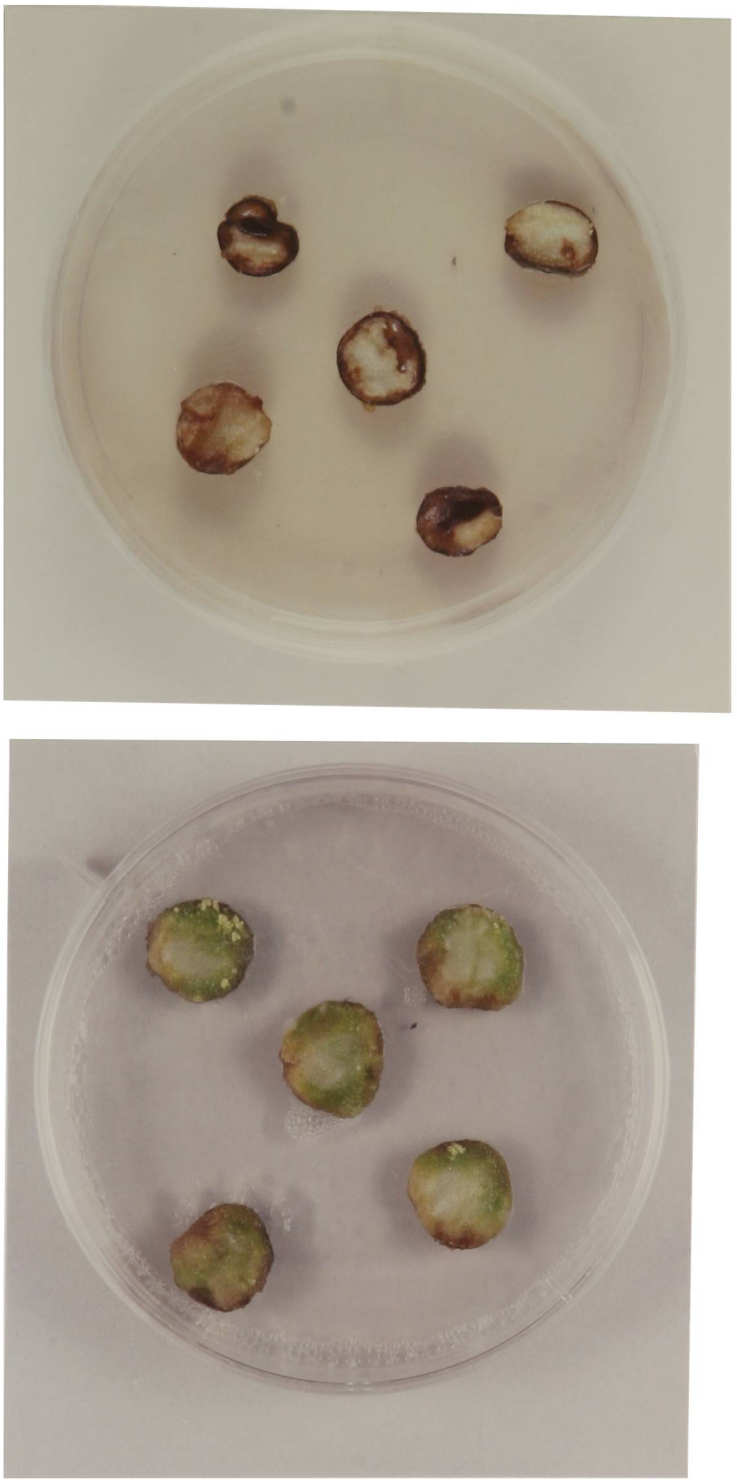


Fig. 6.11 Anther-derived leaf-disc explants after four months incubation at 10±1°C. a: Untreated tissue. b: Leaf-discs treated with 1% EMS for ½h prior to incubation. NOte the presence of small shoot on four out of the five explants (x0.9).

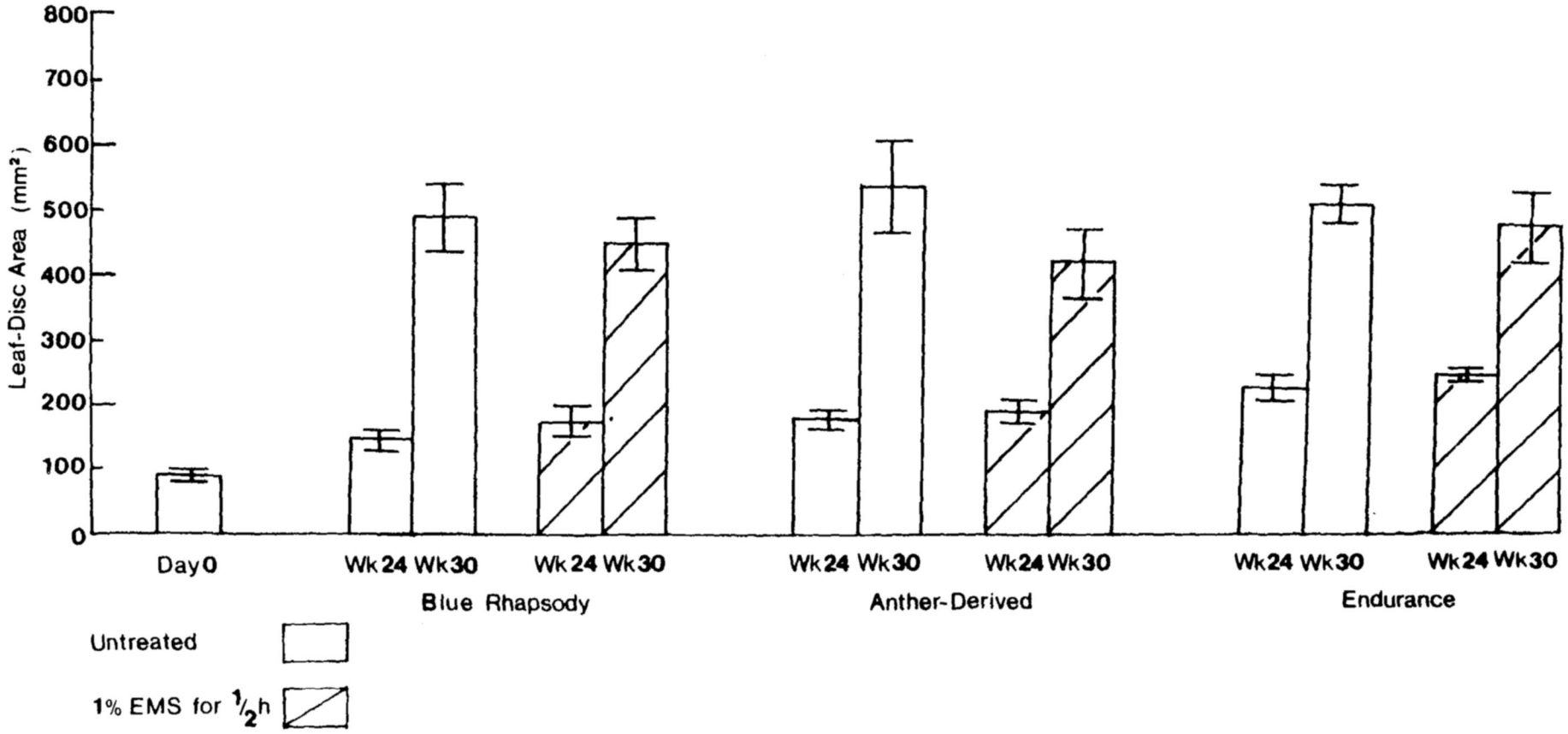


Fig 6.12 Growth of Leaf-Disc Cultures Following Low Temperature Stress. Following incubation at 10°C for six months surviving untreated or EMS treated (1%, ½h) leaf-discs or portions of such discs were transferred to 25°C. Regeneration was determined by measurement of explant area at Wk 24 and 30. Fifty explants were used in each treatment. The experiment was duplicated. Standard errors are indicated.

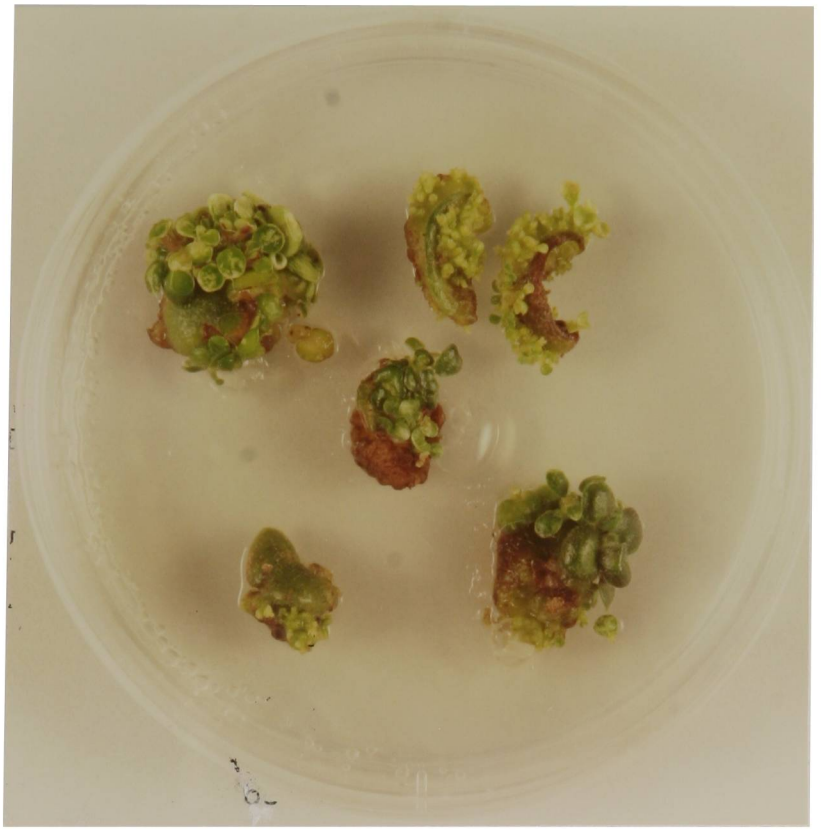
6.3.5 In-Vitro Selection and Regeneration of Cold-Tolerant Plants

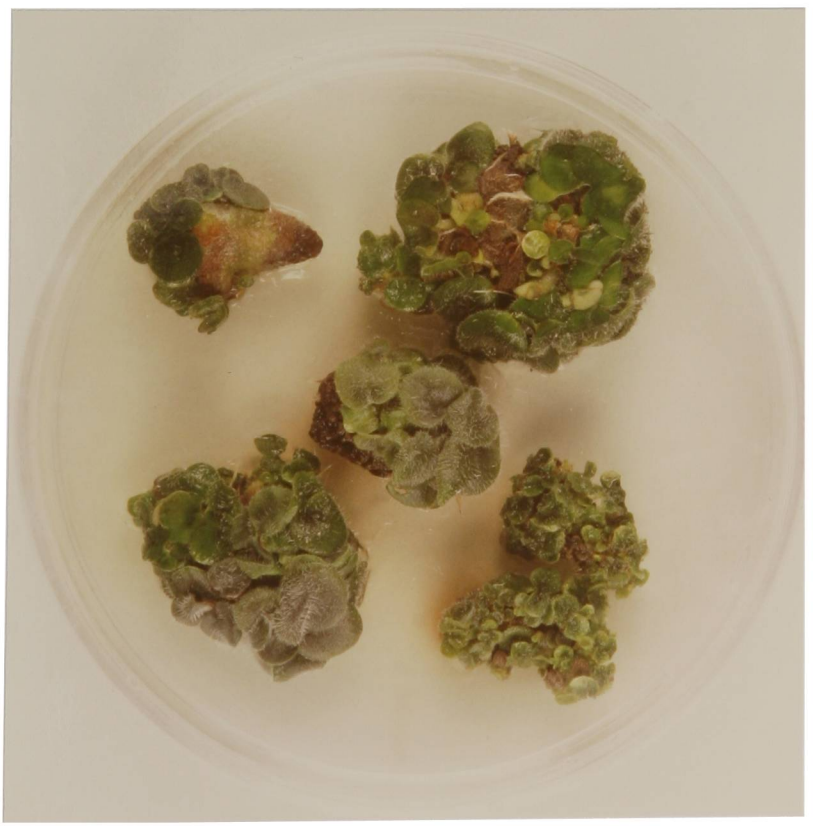
Plant tissues which tolerated 10°C for 6 months were deemed to be cold tolerant. Therefore following 6 months incubated at 10°C leaf-disc cultures or surviving portions of such explants were transferred to 25°C (Fig 6.13a). After six weeks both control and EMS treated leaf-discs of the three Saintpaulia lines under investigation had successfully regenerated shoots (Fig 6.12 and 6.13). The regeneration rate, as measured by increase in explant area (Fig 6.12), of untreated anther-derived Blue Rhapsody disc tissue was 23% and 88% greater than both the Blue Rhapsody parent and Endurance lines respectively (Fig 6.12). All EMS treated leaf-disc tissue regenerated at a slower rate when compared to the untreated regeneration data (Fig 6.12).

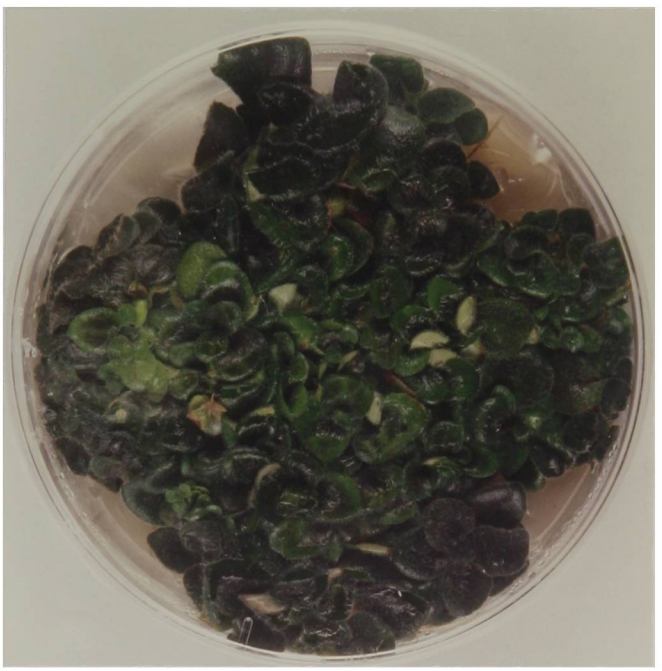
At week thirty single adventitious shoots selected from the above regnerated explants and cultured on basal MS medium at 25°C (Fig 6.13 c and d). After 8 weeks explants were transferred to compost (Fig 6.13f) and grown for a further 8 weeks (Fig 6.13g). These were subseuqnetly tested for cold temperature tolerance (6.3.6).

6.3.6 Growth Analysis of African Violet Plants Selected In-Vitro for Cold Tolerance

Contolr and EMS treated itssue tolerant to 10°C for 6 months was regenerated into plants that were tested for cold temperature tolerance by transfer to environmental cabinets (10 ± 1°C) for 12 weeks. Growth parameters of eight week old plants used in the cold-tolerant screen were determined prior to incubation at 10°C.







Figs. 6.14-6.20 Growth analysis of African violet plants selected in-vitro for cold tolerance.

Control or EMS treated (1%, ½h) tissue tolerant to 10°C for 6 months was regenerated into plants that were transferred to compost and grown for 8 weeks. Subsequently plants were tested for col- tolerance by transfer to environmental cabinets (10±1°C) for 12 weeks. Light was upplied by 36W fluorescent tubes of an intensity of 60uEm^{-2}s^{-1} (10cm from the plants). The photoperiod was 16h light followed by 8h darkness. Three lines were tested; Blue Rhapsody (diploid and anther-derived) and an existing cold tolerant variety, Endurance. The following characteristics were measured for each plant at day 0 and/or week 12: plant diameter (Fig. 6.14), leaf and inflorescence numbers (Figs. 6.15 and 6.19 respectively) and the frequency of chlorosis (number of leaves per plant showing chlorotic symptoms - 6.17). At day 0 and week 12 plants were harvested and the total dry weights of leaf/shoot (Fig. 6.16), roots (Fig. 6.18) and flower stalks (Fig. 6.20) were determined for each plant. Ten to fifteen plants were used in each treatment. Mean and standard errors are indicated.

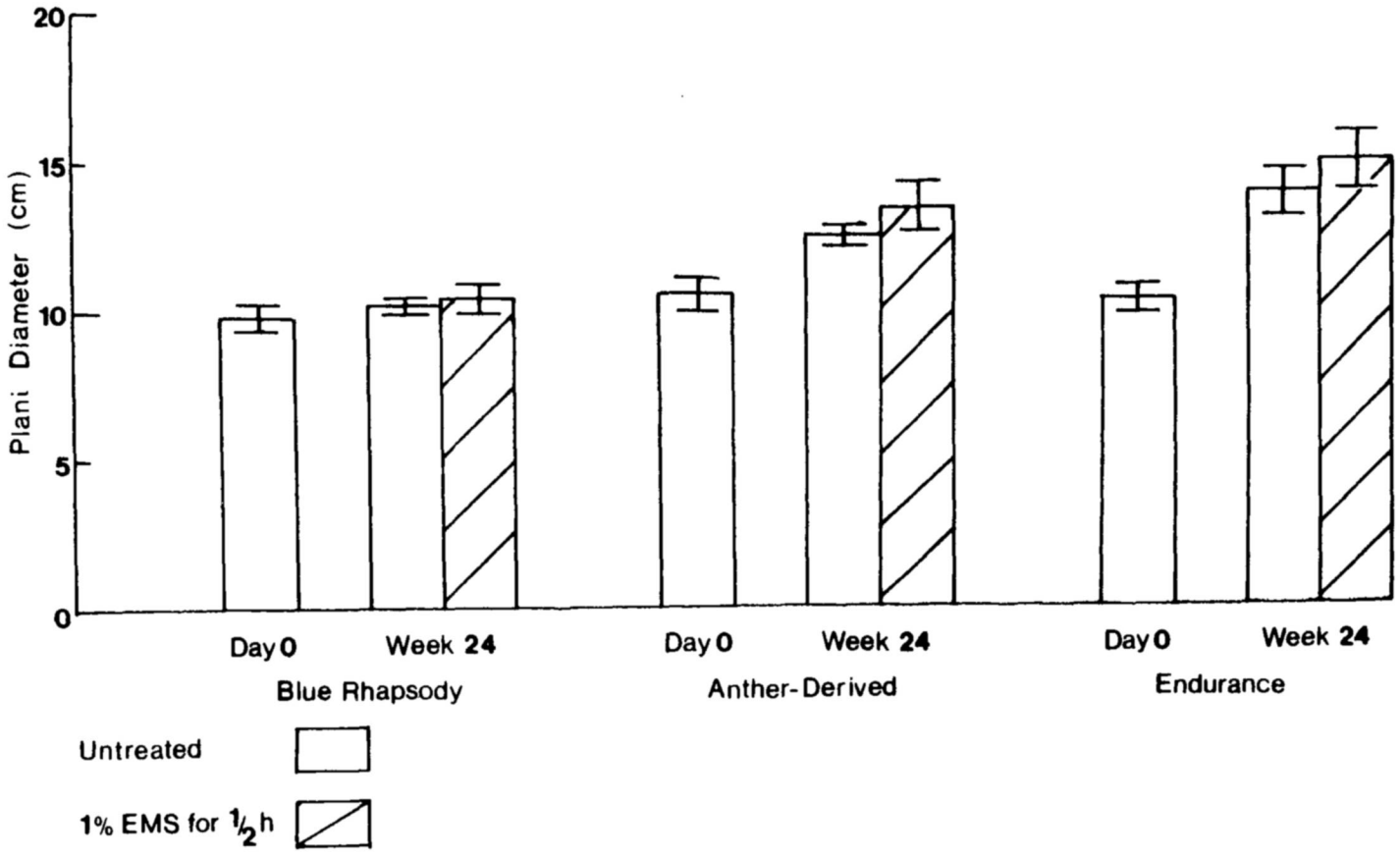


Fig. 6.14 Growth of African Violet Plants Selected <u>In-Vitro</u> for Cold Tolerance

Subsequently growth and flowering data was measured after 12 weeks of treatment (Figs 6.14 - 6.20 inclusive).

Data from the untreated (ctonrol plants) represented inFig 6.14 (plant diamter), 6.15 (leaf production) and Fig 6.16 (leaf/shoot biomass) show essentially the same pattern of growth for the three Saintpaulia lines being screened. The growth rate of anther-derived Blue Rhapsody palnts is more than twice that of the diploid plant (Fig 6.16). This is due to a 30% greater increase in leaf production (Fig 6.15) and 40% drop in leaf chlorosis (Fig 6.17). Comparison of the Blue Rhapsody diploid and anther-derived lines with the cole-tolerant line Endurance shows that both anther-derived and Endurance plants respectively produced 114% and 167% more tissue than the Blue Rhapsody parent (Fig 6.16). Comparison of the leaf biomass of Endurance and anther-derived plants after incubation at $10^{o}C$ for 12 weeks indicated that Endurance produced 25% more tissue than anther-derived lines (Fig 6.16). Endurance lines on average had 10% less chlorotic symptoms than anther-derived plants. However this drop was not signficantly different (Fig 6.17)½

The three Saintpaulia lines investigated showed a 3-4.7 times increase in root dry weight after 12 weeks incubation at $10\pm1^{o}C$ (Fig 6.18). Endurance plants produced significantly more roots than the Blue Rhapsody parent, however, anther-derived lines did not (Fig 6.18).

Whereas, Blue Rhapsody diploid pants did not produce any flower stalks after 12 weeks incubation at $10\pm1^{o}C$, anther-derived lines produced on average

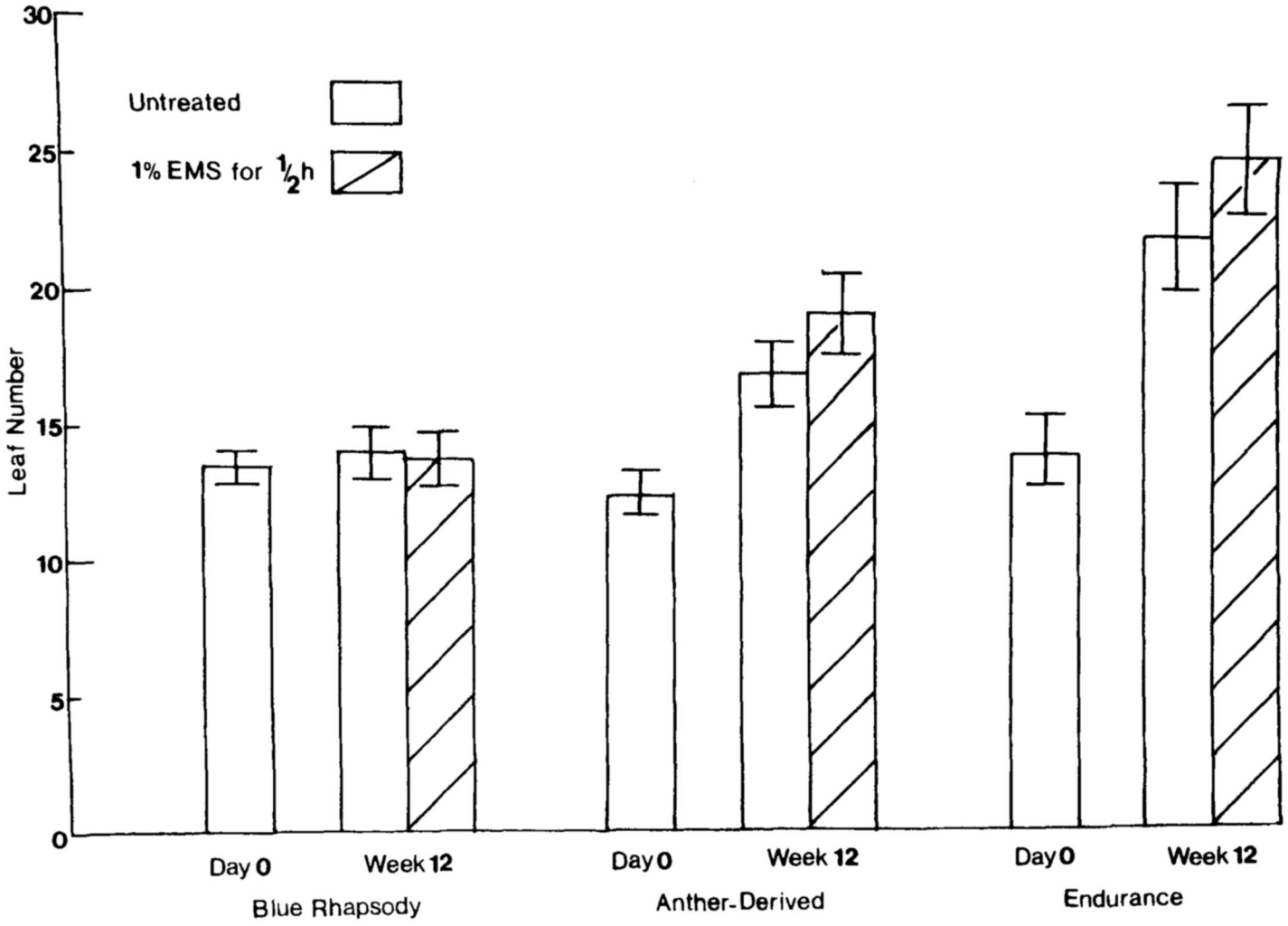


Fig. 6.15 Leaf Production of Arican Violet Selected In-Vitro for Cold Tolerance

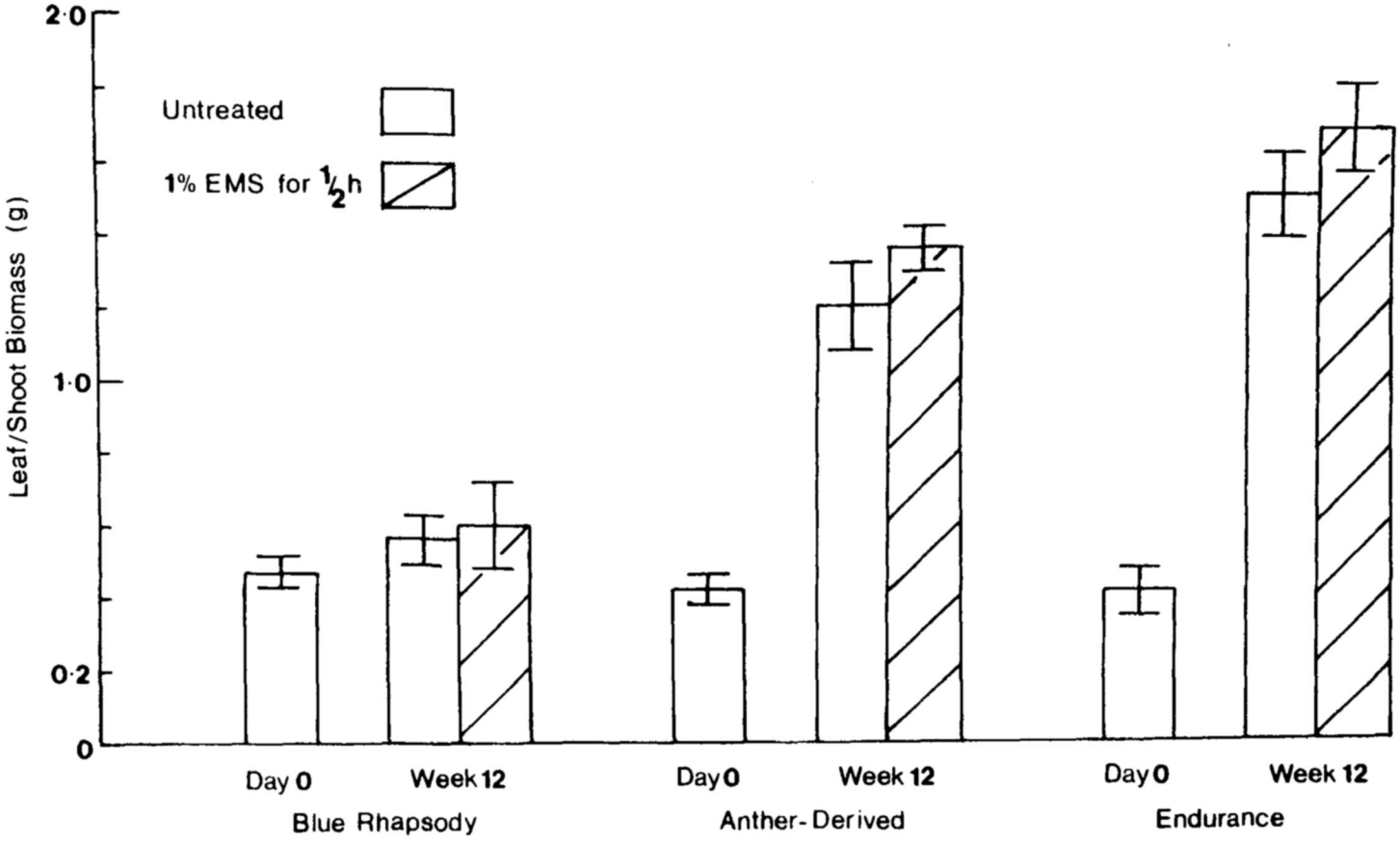


Fig. 6.16 Leaf/Shoot Biomass of African Violet Plants Selected In-Vitro for Cold Tolerance

1.25±0.4(Fig6.19). However, Endurance plants produced twice as many flower stalks, and a seven fold increase in stalk biomass compared to anther-derived lines (Fig 6.20).

After 12 weeks incubation at 10±1°C Blue Rhapsody (diploid and anther-derived lines) and Endurance plants regenerated from tissue treated with EMS showed similar changes in plant diameter (Fig 6.14), leaf production (Fig 6.15) leaf/shoot biomass (Fig 6.16) and leaf chlorosis (Fig 6.17) as those plants regenerated from untreated (control) leaf-discs.

Similar fluctuations in root production were observed in the three Saintpaulia lines initiated from EMS treated discs when compared to control plant material (Fig 6.18). Furthermore, flower stalk production in anther-derived and Endurance lines was the same in both the lines derived from EMS treated and untreated leaf-discs, after 12 weeks incubation at 10±1°C (Figs 6.19 and 6.20).

6.4 Discussion

Incubation of leaf-discs from three lines of Saintpaulia ionantha at 10°C caused a higher degree of electrolyte leakage from Blue Rhapsody parent discs compared to either Blue Rhapsody anther-derived or the Endurance lines. Previous workers have shown that low temperature stress results in membrance damage and leakage of cellular electrolytes (Dexter, et al, 1932; Flint, et al, 1967). This suggests that both anther-derived Blue Rhapsody and Endurance lines are more tolerant to low temperature than the Blue Rhapsody diploid line. The conductivity method has been used with considerable success in a number of crop species to distinguish between cultivars which differ only slightly in cold tolerance (strawberries: Lapin, 1962; Solanum: Sukumaran and Weiser, 1972). Therefore by application of

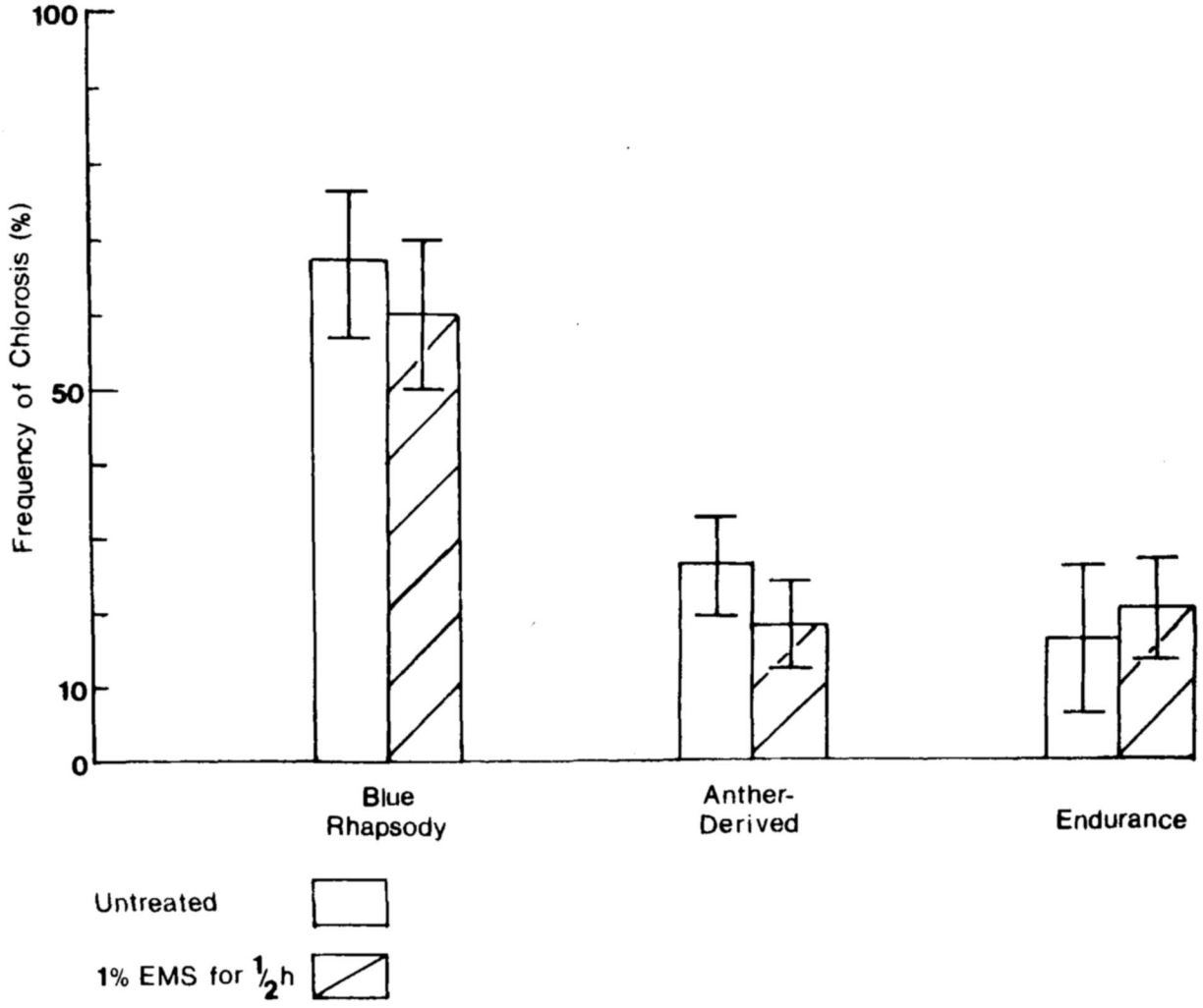


Fig. 6.17 Frequency of Chlorosis of African Violets Selected In-Vitro for Cold Tolerance

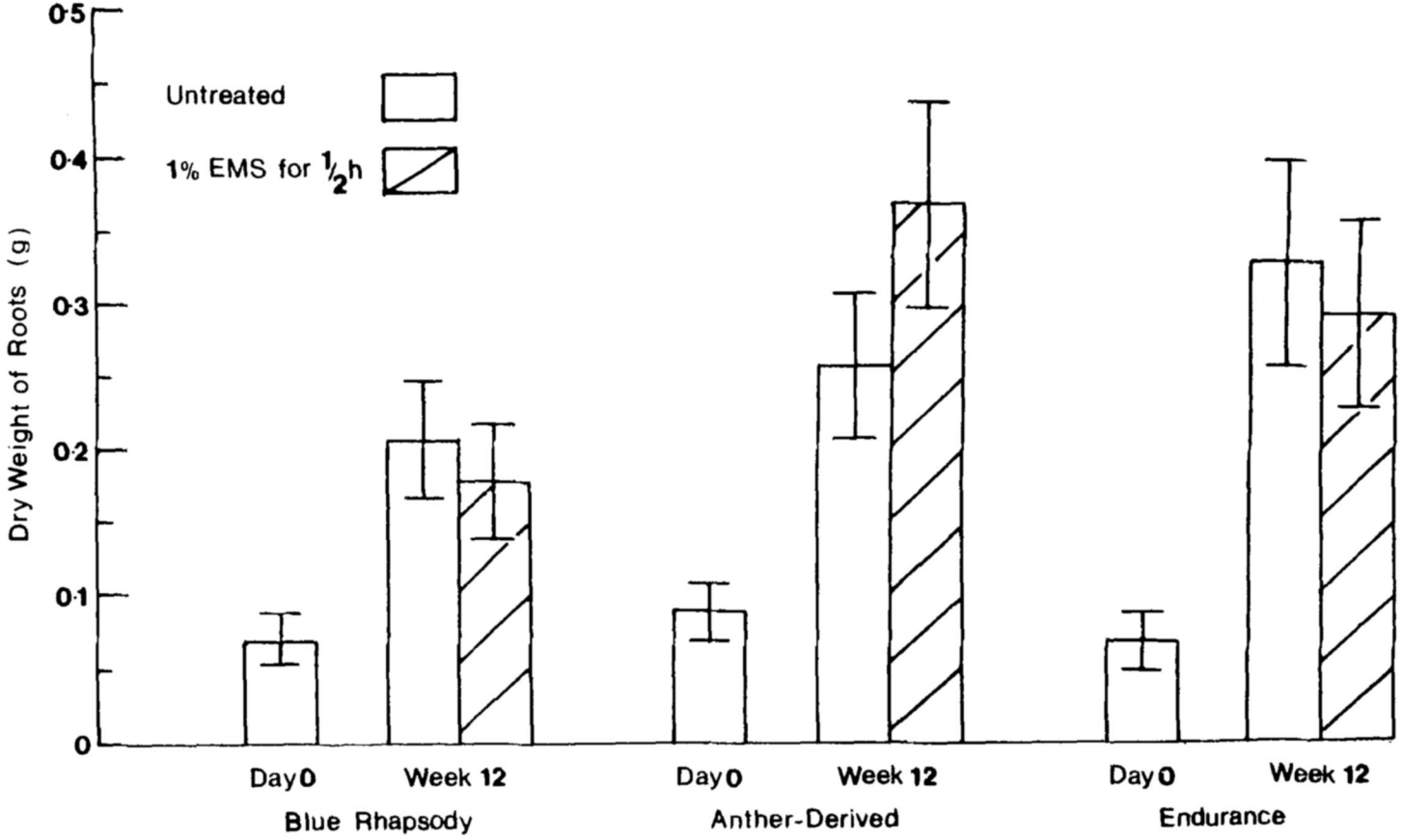


Fig. 6.18 Root Growth of African Violet Plants Selected <u>In-Vitro</u> for Cold Tolerance

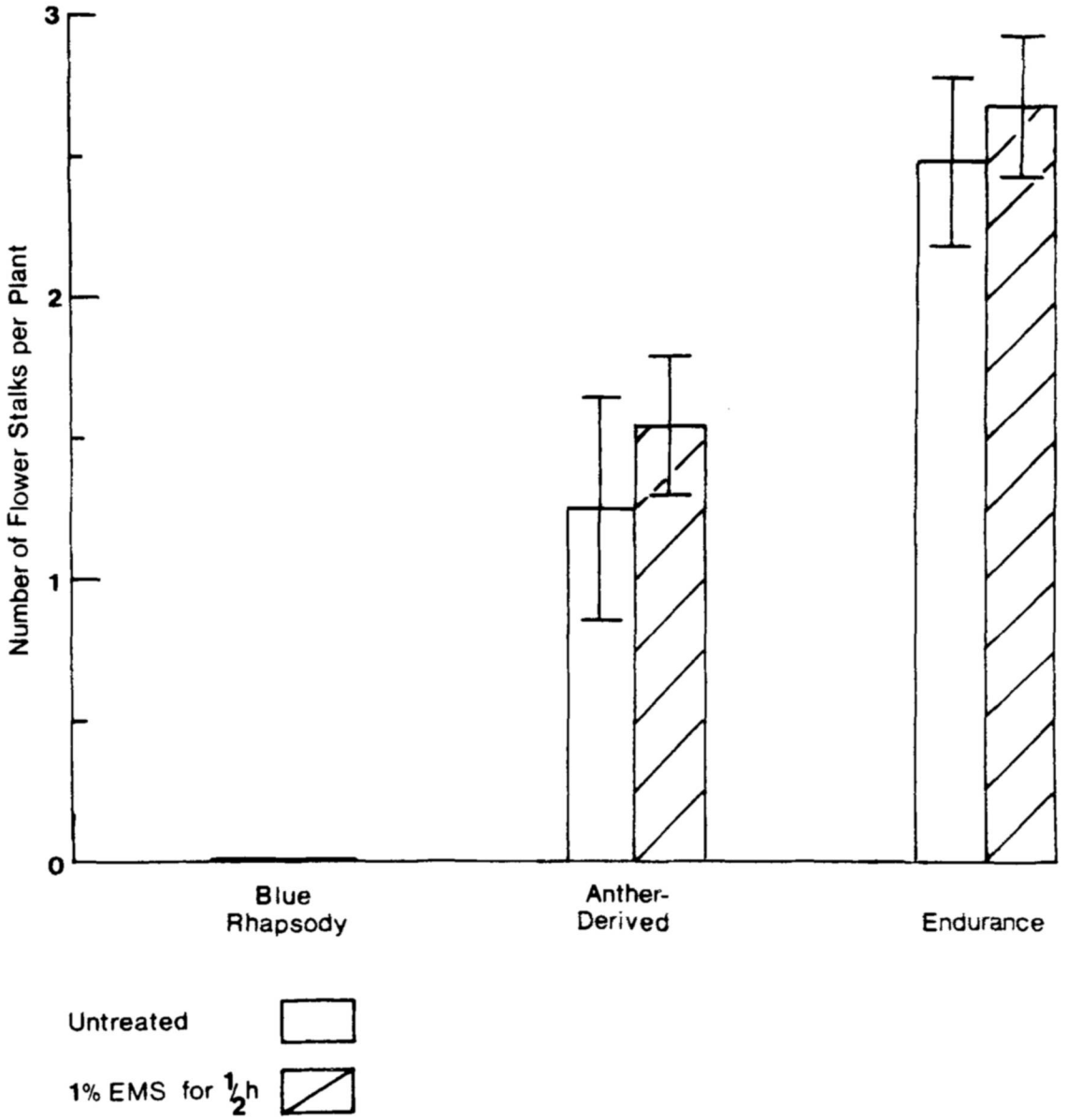


Fig. 6.19 Flower Stalk Production of African Violet Plants Selected <u>In-Vitro</u> for Cold Tolerance

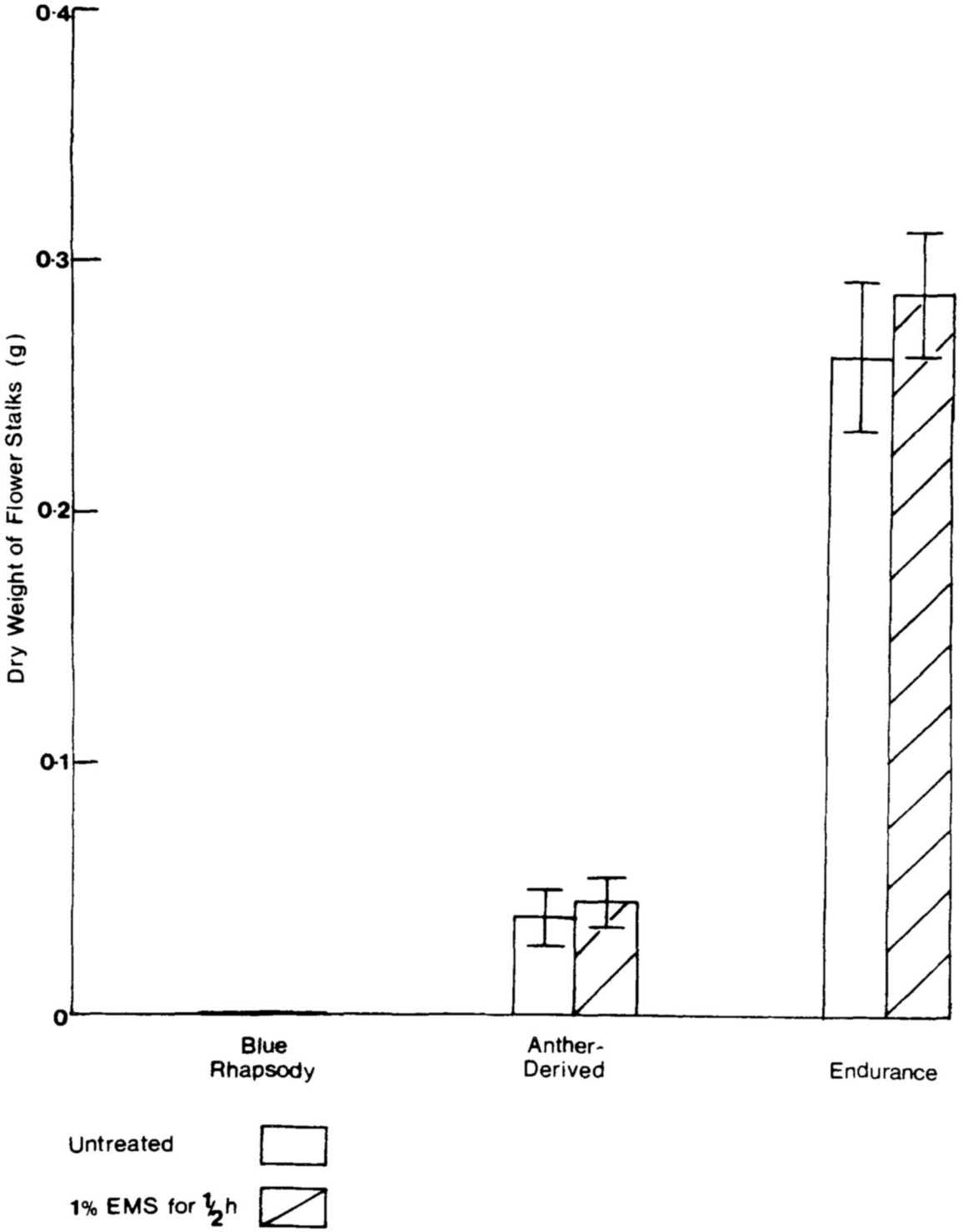


Fig. 6.20 Flower Stalk Biomass of African Violet Plants Selected In-Vitro for Cold Tolerance

this relatively simple, quick, quantitative and highly reproducible method to three lines of the chill sensitive species Saintpaulia ionantha it has been possible to separate the more cold tolerant lines (anther-derived and Endurance) from the more cold sensitive line (Blue Rhapsody diploid). Furthermore, incubation of leaf-discs, from the three African violet lines, at 5°C causes a steep rise in electrolyte leaking indicating the lower limit in which to screen for cold tolerance.

Previous workers have obtained mutants from in-vitro or in-vivo cultured Saintpaulia leaf tissue using the mutagen EMS (for 30 min or 1h) at concentrations ranging from 1-5% (Warfield, 1973; Geier, 1983). The mutants obtained in these studies were accomplished with zero lethality to the treated leaf tissue. However, in this zero lethality was never recorded as exposure of leaf-discs with EMS caused necrosis in the majority of leaf-discs treated.

Compared to conventional Saintpaulia mutation breeding programmes using leaf cuttings (Warfield, 1973; Hentrich, et al, 1974; Polheim, et al, 1974) much higher number of mutants could be recovered from tissue cultures. The total number of mutants and, more importantly, the number of different mutants, which can be recovered from a single culture is dependent on the frequency and type of regeneration. Callus proliferation with subsequent multiple shoot formation may restrict genetic diversity, whereas direct development of single shoots from a single cell of high frequency ensures recovery of a smany different mutant genotypes as possible. The latter type of regeneration, therefore should be favoured by appropriate adjustment of growth regulators.

Undoubtedly, high capacity for regeneration is highly important in mutation breeding, however full advantage of cell and tissue culture systems can only be taken if selection for desired genotypes is possible in-vitro. If mutant genotypes are expressed at the cellular level these systems provide enormously large populations being available to selection. Work by Geier (1983) has shown that the epidermis of a single Saintpaulia leaf segment of 64mm^2 is composed of 10,000-20,000 cells. Furthermore, Geier (1983) found that most of the epidermal cells can be induced to divide but due to competition effects relatively few finally develop into shoots.

The selectron procedure adopted, required untreated or mutagen treated leaf-disc tissue to survive and produce shoots after six months incubation at 10^{o}C. Thereafter, cultures were returned to 25^{o}C leading to the rapid development and regeneration of plants from surviving shoots. Plants were then screened for cold tolerance. The results reported in this study confirm Geiers (1983) earlier observations that incubation of cultured leaf lamina at 5^{o}C proved to be lethal. However, the results show that small areas, of leaf-discs excised from the three different lines of Saintpaulia survived and produced adventitious shoots after six months incubation at 10^{o}C. This contrasts with data of Schlegel (1982) and Geier (1983) who reported that temperatures below 15^{o}C did not permit any growth of African violet explants.

The application of EMS to cultured leaf-discs of the three African violet lines, prior to incubation at 10^{o}C for six months, did not significantly increase their viability, biomass and shoot production compared to control tissue. Furthermore, all plantlets regenerated from treated leaf-discs turned out

to be non-chimeric suggesting that they are either completely uneffected by the mutagen or that they are solid mutants. Enhanced chilling resistance of cell lines selected in-vitro was found to be associated with altered mitochondrial characters (Dix, et al, 1976). Geier (1983) found that 1% EMS was especially effective in inducing plastome mutations in Saintpaulia and inferred that this mutagen was also effective in inducing mutations in mitochondrial genes and therefore might be useful with regard to the induction of low-temperature mutants. However,no such plastome mutations were recorded in this study suggesting that EMS was ineffective.

Results from this study show that Blue Rhapsody anther-derived plants performed significantly better $10^{o}C$ when compared to the diploid parent. Therefore, it is considered that the anther-derived lines are real physiological mutants and are not epigenetic variants (non-heritable phenotypes with modified physiological response).

CHAPTER SEVEN

GENERAL DISCUSSION

General Discussion

The interest in haploids originates largely from their considerable potential for plant breeding (Kasha, 1974; Reiniert and Bajaj, 1977; Bajaj, 1983). Haploids can be used to facilitate the detection of mutations and the recovery of unique recombinants. Since most mutations are recessive, they are difficult to detect. However, haploids possess only one set of alleles at each locus, therefore it is possible for recessive mutants to be detected. Haploids can be useful not only in obtaining mutants at a much higher frequency but doubling of the chromosome number of such lines offers a method for the rapid production of homobygous plants. These in turn may be use for producing inbred lines for hybrid production.

The culture of African violet (cv Blue Rhapsody) anthers may be successfully achieved as is shown in the experiments described in Chapter 3. Furthermore, work in this chapter elaborates upon the earlier observations of Weatherhead, et al, (1982). Using the same commercial line described by these workers, a correlation between pollen development stage and bud diameter was found. Thus it is probably that a similar correlation could be made with other African violet varieties. An updated protocol is recommended to optimise callus, induction, production and shoot proliferation from African violet anthers. it is advised that Blue Rhapsody anther donor plants should be grown at a constant growing temperature of 25^{o}C. Intact anthers should then be excised from buds greater than 5mm in diameter (uninucleate, stage 3; mitotic, stage 4 and binucleate, stage 5) and plated on solid MS medium supplemented with NAA:BAP; 1:0.5mgl^{-1} (Weatherhead, et al, 1982). To optimise morphogenesis all cultures should be incubated in the light initially at 15^{o}C

(14d) and then transferred to 25°C for 28d. Further multiplication shoots onto MS medium supplemented with NAA:BAP; 1:1mgl^{-1}. Thsu Blue Rhapsody anthers cultured on the medium of Weatherhead, et al, (1982) readily produce callus of a highly morphogenic nature and therefore high propagation potential. However, evidence from this investigation shows that the callus produced is essentially derived from the somatic tissue (anther wall and connective) of the anther. To circumvent the problem of callusing somatic tissue, isolated pollen was cultured on an anther nurse tissue. Results from these experiments revealed that Blue Rhapsody pollen has a division capability, thus highlighting the most promising approach for future studies to obtain pollen callus and/or embryoids on a regular basis.

The data reported in Chapter 4 supports the above view that the majority of plants regenerated from anther callus are derived from the somatic tissues of the anther. Anthers cultured using either the method of Hughes, et al (1975) or Weatherhead, et al (1982) produced diploid and aneuploid plants. The incidence of non-haploidy is common in anther culture research. Previous work has shown that non-haploidy is prevalent in both embryogenic and callus producing species and does not appear to be related to the hormonal requirements for androgenesis (Vasil, 1980). Furthermore, diploid as well as aneuploid plants are formed particularly when older anthers are cultured (Kasperbauer and Collins, 1972; Engvild, 1974).

The production of haploid African violet plants has often proved to be problematic and unreliable. Weatherhead, et al (1982) reported 24% non haploids from their regenerated anther-derived plants and suggested a somatic origin for these lines. Later

work by Khokhar (1983) was unable to repeat haploid production as reported by Weatherhead, et al (1982). The data from this investigation re-emphasises the necessity for rigorous screening of regenerants to ensure haploidy before plants are included in breeding programmes. After such screening in this study the following conclusions were made. The morphological variation, observed in the majority of plant lines derived from anther callus is by definition somaclonal in nature (larkin and Scowcroft, 1981). Furthermore, due to the high incidence of aneuploidy it is highly probably that the observed variation has a genetic basis (Bajaj, 1983). However, it is proposed that one plant, namely the miniature (Chapter 3) is of possible pollen origin and has undergone spontaneous doubling in the course of regeneration and subsequent growth. Doubling in African violet haploids have been previously reported (Bhaskaran, et al, 1983).

The studies in Chapter 5 lead to three main conclusions. Firstly, after screening for low temperature tolerance it was observed that the anther-derived somaclonal variants can survive temperatures as low as 15^{o}C. Furthermore, such lines are of marketable quality. Secondly, work in this thesis confirms the data of Bilkey (1981), that the existing cold tolerant line, Endurance, produced by sexual hybridization thrives and flowers in abundance at low temperatures. Finally, it was not possible to exploit physiological adaption and therefore to grow African violets at low temperatures and produce plants of marketable quality by extending the photoperiods described by Went (1959,1960).

The evidence presented in Chapter 6 (on the in-vitro and in-vivo screening of the three plant lines at low temperature) confirms the above observation that Blue Rhapsody anther-derived lines can tolerate and flourish at low temperatures.

Distinction between genetic and epigenetic variants is probably the most serious problem in screening tissue cultures for resistant mutants (Geier, 1983). Furthermore, hereditary transmission of altered character in variants recovered from cell and tissue cultures has been proved in only relatively few cases (Reisch, 1983). In this study no evidence is presented to show that the cold tolerant trait was sexually transmittable. However, taken into consideration that a cold tolerant African violet already exists (and has successfully proven to be as such in this study) in conjunction with the evidence from this work that anther-derived lines always performed significantly better than the Blue Rhapsody parent plants the following conclusions can be made. That after three separate screenings (Chapter 5 and 6), in vivo and in vitro at an extreme temperature of $10^{o}C$; it is considered that the above lines are genuine physiological mutants and not epigenetic variants (non-heritable phenotypes with modified physiological response). Furthermore, Geier (1983) proposed a selection protocol of alternate periods of stress and optimal temperatures to restrict physiological adaption and thereby result in a clear separation of mutant and epigenetic variants. Such criterion were observed in this study.

Work in this investigation has also shown that leaf-disc explants can be used as an effective means of obtaining a population of cells for further

in-vitro selection studies in African violet. However, evidence suggests levels of EMS higher than 1% should be used to obtain effective mutagenesis in such a system.

Furthermore, leaf-disc tissue treated with mutagen did not show a significantly higher rate of survival than untreated tissue after 12 and 24 weeks incubation at 10°C (Figs 6.7 and 6.8). Nonetheless what is significant from this series of experiments is that on average between 7 and 29% of untreated leaf-disc tissue was not necrotic after 24 weeks incubation at 10°C. Furthermore, the anther-derived line has significantly (11%) less necrotic tissue per leaf-disc than the Blue Rhapsody anther-derived line. However, Endurance leaf-discs have the lowest frequency of necrosis recorded (71%) (Fig 6.8). Pretreatment of African-violet leaf-disc tissue with 1% EMS for ½h prior to incubation at 10°C does not significantly decrease the frequency of necrosis per disc (Fig 6.8).

REFERENCES

ABO, EL-NIL. and HILDEBRANDT, A.C. (1972). Morphological changes in geranium plants differentiated from anther cultures. In vitro 7, 258 (abstr.).

ABO, EL-NIL, M.M. and HILDEBRANDT, A.C. (1973). Origin of androgenic callus and haploid geranium plants. Can. J., Bot. 51, 2107-2109.

ANAGNOSTAKIS, S.L. (1974). Haploid plants from anthers of tobacco-enhancement with charcoal. Planta 115, 281-283.

ANONYMOUS. (1980). The Grower 93 (2), 4.

ARISUMI, T. (1964). Interspecific hybriduzation in African violets. J. Hered. 55, 181-183.

ARTHUR, J.M. and HARVILL, E.K. (1937). Plant growth under continuous illumination from sodium lamps supplemented by mercury arc lamps. Boyce Thompson Inst. Contrib. 8, 433-443.

BABBAR, S.B. and GUPTA, S.C. (1982). Promotory effect of polyvinylpyrrolidone and L-Cysteine-HCl on pollen plantlet production in anther cultures of Datura metel. Z. Pflanzenphysiol 106, 459-464.

BAJAJ, Y.P.S. (1970). Effect of gamma-irradiation on growth, RNA, protein and nitrogen contents of bean callus cultures. Ann. Bot. 34, 1089-1096.

BAJAJ, Y.P.S., REINERT, J. and HEBERLE, E. (1977). Factors enhancing in vitro production of haploid plants in anthers and isolated microspores. In: La Culture des Tissue et des Cellules des Vegetaux (R.J. Gautheret, ed.) pp 47-58. Mason Press, Paris, New York.

BAJAJ, Y.P.S., RAM, A.K., LABANA, K.S. and SINGH, H. (1981). Regeneration of genetically variable plants from the anther-derived callus of Arachis hypogaea and Arachis villosa. Plant Sci. Lett. 23, 35-39.

BAJAJ, Y.P.S. (1983). In vitro production of haploids. In: D.A. Evans, W.R. Sharp, P.V. Ammirato and Y. Yamada (Eds.), Handbook of plant cell culture, Vol 1. pp 228-287. Macmillan Publishing Co., New York.

BAYLISS, M.W. (1980). Chromosomal variation in plant tissues in culture. Intern. Rev. Cytol. Suppl. 11B. 113-144.

BEHNKE, M. (1979). Selection of potato callus for resistance to culture filtrates of Phytophthora infestans and regeneration of resistant plants. Theor. Appl. Genet. 55, 69-71.

BENNICI, A. (1974). Cytological analysis of roots, shoots and plants regenerated from suspension and solid in-vitro cultures of haploid Pelargonium. Z. Pflanzenzuchtg. 72, 199-205.

BHASKARAN, S., SMITH, R.H. and FINER, J.J. (1983). Ribulose bisphosphate carboxylase activity in anther-derived plants of Saintpaulia ionanther Wendl. Shag. Plant Physiol 73,639-642.

BIDNEY, D.L. and SHEPHARD, J.F. (1981). Phenotypic variation in plants regenerated from protoplasts: the potato system. Biotech. Bioeng. 23, 2691-2701.

BILKEY, P.C., McCOWN, B.H. and HILDEBRANDT, A.C. (1978). Micropropagation of African violet from petiole cross-sections. Hortscience 13, 37-38.

BILKEY, P.C. (1981). An assessment of the suitability of Saintpaulia for plant genetic manipulations. PhD. thesis. University of Nottingham, Nottingham.

BILKEY, P.C. and COCKING, E.C. (1981). Increased plant vigour by in vitro propagation of Saintpaulia ionanther Wendl. from Sub-epidermal tissue. Hortscience 16, 643-644.

BILKEY, P.C. and COCKING, E.C. (1982). A non-enzymatic method for the isolation of protoplasts from callus of Saintpaulia ionantha African violet. Z. Pflanzenphysiol 105, 285-288.

BILKEY, P.C., DAVEY, M.R. and COCKING, E.C. (1982). Isolation, origin and properties of enucleate plant microplasts. Protoplasma 110, 147-151.

BLAYDES, D. (1966). Interaction of Kinetin and various inhibitors in the growth of soybean tissue. Physiol. Plant. 19, 748-753.

BOURGIN, J.-P. and NITSCH, J.P. (1967). Obtention de Nicotiana haploides a partir d'etamines cultivees in vitro Ann. Physiol. Veg. 9, 377-382.

BROERTJES, C., HACCIUS, B. and WEIDLICH, C. (1968). Adventitious bud formation on isolated leaves and its significance for mutation breeding. Euphytica 17, 321-344.

BROERTJES, C. (1968). Dose rate effects in Saintpaulia. Mutations in Plant Breeding Z (Proc. Panel. Vienna, 1967) IAEA, Vienna pp. 63-71.

BROERTJES, C. (1969a). Mutation breeding of streptocarpus. Euphytica 18, 333-339.

BROERTJES, C. (1969c). Mutation breeding of Achimenes. Euphytica 21, 48-62.

BROERTJES, C. (1972). Use in plant breeding of acute, chronic or fractionated doses of X-rays or fast neutrons as illustrated with leaves of Saintpaulia. Agric. Res. Rep. 776, Pudoc, Wageningen (Netherlands). 74p.

BROERTJES, C. and VANHARTEN, A.M. (1978). Application of mutation breeding methods in the improvement of vegetatively propagated crops. An interpretative literature review. Elsevier scientific Publ. Co., Amsterdam; pp 316.

BROERTJES, C., KOENE, P. and PRONK, T.H. (1983). Radiation-induced low-temperature tolerant cultivars of Chrysanthemum morifolium Ram. Euphytica 32, 97-101.

BUTISAN, S. Pre-emergence weed control in oilseed rape. In: "Plant Tissue and Cell Culture", (ed., H.E. Street), Blackwell Scientific Publications, Oxford, 177-205.

CASSELLS, A.C. and PLUNKETT, A. (1984). Production and growth analysis of plants from leaf cuttings, and from tissue cultures of disks from mature leaves and young axenic leaves of African violet (Saintpaulia ionantha Wendl.). Scient. Hort. 23, 361-369.

CHAUDHARI, H.K. and BARROW, J.R. (1975). Identification of cotton haploids by stomatal chloroplast-count technique. Crop Science 15, 760-763.

CHEN, C.C. and LIN, M.H. (1976). Induction of rice plantlets from anther culture. Bot. Bull. Acad. Sinica 17, 18-24.

CHEN, C.C. (1977). In vitro development of plants from microspores of rice. In Vitro. 13, 484-489.

CHEN, Z., QIAN, C., QIN, M., WANG, C., SUO, C., CHEN, F. and DENG, Z. (1979). The induction of pollen plants of sugarcane Annual Report of the Institute of Genetics. Academia Sinica, 91-93.

CHEN, Y., WANG, R., TIAN, W., ZUO, Q., ZENG, S., LU, D. and ZHANG,G. (1980). Studies on pollen culture in vitro and induction of plantlets of Oryza sativa L. In D.R. Davies and D.A. Hopwood (eds.). Proc. Fourth John Innes Symp. and second international haploid conference - the plant genome p. 245. John Innes Institute, Norwich.

CHEN, W.H., COCKBURN, W. and STREET, H.E. (1982). Cell plating and selection of cold-tolerant cell lines in sugarcane. Proc. 5th Intl. Cong. Plant Tissue and Cell Culture. pp 485-486.

CHEN, Z., QIAN, C., QIN, M., XU, X. and XIAO, Y. (1982). Recent advances in anther culture of Hevea brasiliensis (Muell.-Arg). Theor. Appli. Genet. 62, 103-108.

CHU, C-C., WANG, C-C., SUN, C., HSU, C., YIN, K-C., CHU, C-Y. and BI, F-Y. (1975). Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci. Sin. 18, 659-668.

CHAUNG, C-C., OUYANG, T-W., HSU, C., CHOU, S-M. and CHING, C-K. (1978). A set of potato media for wheat anther culture. In: Proceedings of Symposium on Plant Tissue Culture, Science Press, Peking, pp 51-56.

CLAPHAM, D. (1971). In vitro development of callus from the pollen of Lolium and Hordeum. Z. Pflanzenzücht. 65, 285-292.

CLAPHAM, D. (1973). Haploid Hordeum plants from anthers in vitro. Z. Pflanzenzucht 69, 142-155.

CLAPHAM, D. (1977). Haploid induction in cereals. In: J. Reinert and Y.P.S. Bajaj (eds.), Plant cell, tissue, and organ culture, pp. 279-298. Springer-Verlag, Berlin.

CLAYBERG, C.D. (1961). Hybridizing with the African violet species. African Violet Magazine. 15, 105-107.

COLHOUN and STEER (1983). The cytological effects of the gametocides Ethrel and RH-531 on microsporogenesis in Barley (Hordeum vulgare L.). Plant, Cell and Environment 6, 21-29.

COOKE, R.C. (1977). Tissue culture propagation of African violets. Hortscience 12, 549.

COLLIN, H. (1984).

COSSETTE, F. and PAUZE, F.J. (1983). La production de lignees d'orge haploides et diploides homozygotes par culture d'antheres. Rev. Can Biol. Exptl. 42, 45-49.

DALE, P.J. and HUMPHREYS, M.W. (1974). Welsh Plant Breed Stn. (Aberystwyth) Rep. No. 88.

DALE, P.J. (1975). Pollen dimorphism and anther culture in barley. Planta 127, 213-220.

D'AMATO, F. (1978). Chromosome number variation in cultured cells and regenerated plants. In: Frontiers of Plant Tissue Culture (ed. Thorpe, T.A.), pp. 287-295. Calgary, Intern Ass. Plant. Tissue. Culture.

DEAMBROGIO, E. and DALE, P.I. (1980). Effect of 2,4-D on the frequency of regenerated plants in barley (Hordeum vulgare) cultivar 'Akka' and on genetic variability between them. Cereal Res. Commun. 8, 417-424.

DEMAGGIO, A.E., WETMORE, R.H., HANNAFORD, J.E., STETLER, D.A. and RAGHAVAN, V. (1971). Ferns as a model for studying polyploidy and gene dosage effects. Bioscience 21, 313-316.

DEVREUX, M. and SACCARDO, F. (1971). Mutazioni sperimentali osservate su plante aploidi di tabacco ottenute per colture in vitro di antere irradiate. Atti. Ass. Genet. Ital. 16, 69-71.

DEXTER, S.T., TOTTINGHAM, W.E. and GRABER, L.F. (1932). Investigations of the hardiness of plants by measurement of electrical conductivity. Plant Physiol. 7, 63-78.

DIX, P.J. and STREET, H.E. (1976). Selection of plant cell lines with enhanced chilling resistance. Ann. Bot. 40, 903-910.

DIX, P.J. (1977). Chilling resistance is not transmitted sexually in plants regenerated in Nicotiana sylvestris cell lines. Z. Pflanzenphysiol. 84, 223-226.

DORE, C. and LAMBERT, A. (1973). La culture d'antheres in vitro. Revue Hort (Paris) 145,44-48.

DUNWELL, J.M. and SUNDERLAND, N. (1973). Anther culture of Solanum tuberosum L. Euphytica 22, 317-323.

DUNWELL, J.M. (1976). A comparative study of environmental and developmental factors which influence embryo induction and growth in cultured anthers of Nicotiana tabacum. Enviro. and Expt. Bot. 16, 109-118.

DUNWELL, J.M. (1979). Anther culture in Nicotiana tabacum: The role of the culture vessel atmosphere in pollen embryo induction and growth. J. Exp. Bot. 30, 419-428.

DUNWELL, J.M. (1981). Stimulation of pollen embryo induction in tobacco by pretreatment of excised anthers in a water-saturated atmosphere. Plant Sci. Lett. 21, 9-13.

EHRLICH, H.G. (1958). Cytological studies in Saintpaulia Wendl. (Gesneriaceae) Amer. J. Bot. 45, 177-182.

ENCHEV, Y. (1976). Induced mutations in winter brewing barley and their use. Barley. Genet. 3, 190-196.

ENGELS, F.M., VAN DER LAAN, F.M., LEENHOUTS, H.P. and CHADWICK, K.H. (1980). The regeneration of epidermal cells of Saintpaulia leaves as a new plant-tissue system for cellular radiation biology. Int. J. Radiat. Biol. 38, 309-321.

ENGVILD, K.C., LINDE-LAURSEN, I. and LUNDQVIST, A. (1972). Anther cultures of Datura innoxia: flower bud stage and embryoid level of ploidy. Hereditas 72, 331-332.

ENGVILD, K.C. (1973). Triploid petunias from anther cultures. Heriditas 74, 144-147.

ENGVILD, K.C. (1974). Plantlet ploidy and flower bud size in tobacco anther cultures. Hereditas 76, 320-322.

ERIKSSON, T. (1965). Studies on the growth requirements of cell cultures of Haplopappus gracilus. Physiol. Plant. 18, 976-993.

ESPINO, F.J. and VAZQUEZ, A.M. (1981). Chromosome numbers of Saintpaulia ionantha plantlets regenerated from leaves cultured in vitro with caffeine and colchicine. Euphytica 30, 847-853.

FITCH, M.M. and MOORE, P.H. (1983). Haploid production from anther culture of Saccharum spontaneum L. Z. Pflanzenphysiol. 109, 197-206.

FITCH, M.M. and MOORE, P.H. (1984). Production of haploid Saccharum spontaneum L. Comparison of media for cold incubation of panicle branches and for float culture of anthers. J. Plant Physiol. 117, 169-178.

FLINT, N.L., BOYCE, B.R. and BEATTIE, D.J. (1967). Index of injury- a useful expression of freezing injury to plant tissues as determined by the electrolyte method. Can. J. Plant. Sci. 47, 229-230.

FLORES, H.E., FIERRO, C.A. and KOO, F.K.S. (1976). Tissue culture of African violet Saintpaulia ionantha Wendl. 24th Annual Conress of the American Society of Horticultural Sciences, Tropical Region.

FORD, A.J. (1953). Root and crown roots of African violets. African Violet Magazine 3, 20-28.

FOROUGHI-WEHR, B. and FRIEDT, W. (1984). Rapid production of recombinant barley yellow mosaic virus resistant Hordeum vulgare lines by anther culture. Theor. Appl. Genet. 67, 377-382.

FRANDSEN, N.O. (1967). Haploidproduktion aus einem Kartoffelzucht-material mit intersiver Wildarteinkreuzung. Der Zuchter 37, 120-134.

FRIEDT, W., LIND, V., WALTHER, H., FOROUGHI-WEHR, B., ZUCHNER, S. and WENZEL, G. (1983). The value of inbred lines derived from Secale cereale x S.vavilovii via classical inbreeding and androgenetic haploids. Z. Pflanzenzuchtg. 91, 89-103.

GAMBORG, O.L., MILLER, R.A. and OJIMA, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50, 151-158.

GARNER, W.W. and ALLARD, H.A. (1920). Effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants. J. Agr. Res. 18, 553-606.

GEIER, T. (1983). Induction and selection of mutants in tissue cultures of Gesneriaceae. Acta. Hort. 131, 329-337.

GENOVESI, A.D. and COLLINS, G.B. (1982). In vitro production of haploid palnts of corn via anther culture. Crop Sci. 22, 1137-1144.

GEORGE, L. and NARAYANASWAMY, S. (1973). Haploid Capsicum through experimental androgenesis. Protoplasma 78, 467-470.

GHARYAL, P.K., RASHID, A. and MAHESHWARI, S.C. (1983). Androgenic response from cultured anthers of a leguminous tree, Cassia siamea Lam. Protoplasma 118, 91-93.

GRESSHOFF, P.M. and DOY, C.H. (1972a). Development and differentiation of haploid Lycopersicon esculentum (tomato). Planta 107, 161-170.

GRESSHOFF, P.M. and DOY, C.H. (1972b). Haploid Arabidopsis thaliana callus and plants from anther culture. Aust. J. Biol. Sci. 25, 259-264.

GRESSHOFF, P.M. and DOY, C.H. (1974). Derivation of a haploid cell line from Vitus vinifera and the importance of the stage of meiotic development of anthers for haploid culture of this and other genera. Z. Pflanzenphysiol. 73, 132-141.

GROUT, B.W.W. and WEATHERHEAD, M.A. (1980). A strategy for the production of disease-resistant mutants. In: Ingram, D.S. and Helgeson (eds.) Blackwell Scientific Publications. pp. 249-254.

GRUNEWALDT, J. (1976). In vitro regeneration of leaf-petiole cross sections from Saintpaulia ionantha H. Wendl. Gartenbau-wissenschaft 41, 145-148.

GRUNEWALDT, J. (1980). Spontaneous and induced colour mottling in Saintpaulia ionantha H. Wendl. Gartenbauwissenschaft 45, 124-128.

GRUNEWALDT, J. (1983). In vitro mutagenesis of Saintpaulia and Pelargonium cultivars Acta. Hort. 131, 339-343.

GUHA, S. and MAHESHWARI, S.C. (1964). In vitro production of embryos from anthers of Datura. Nature 204, 497.

GUHA, S. and MAHESHWARI, S.C. (1966). Cell division and differentiation of embryos in the pollen grains of Datura in vitro. Nature 212, 97-98.

GUHA, S. and MAHESHWARI, S.C. (1967). Development of embryoids from pollen grains of Datura in vitro. Phytomorphology. 17, 454-461.

GUPTA, S. (1976). Morphogenetic response of haploid callus tissue of Pisum sativum (var Bzz). Ind. Agric. 20, 11-21.

GUPTA, P.P. (1982). Genesis of microspore-derived triploid Petunias. Theor. Appl. Genet. 61, 327-331.

HANDRO, W. (1981). Mutagenesis and in vitro selection. In: Thorpe, T.A. (Ed) Plant tissue culture. Methods and applications in agriculture. Academic Press, New York: 155-180.

HARNEY, P.M. and KNAP, A. (1979). A technique for the in vitro propagation of African violets using petioles. Can. J. Plant Sci. 59, 263-266.

HAVRANEK, P. and VAGERA, J. (1979). Regulation of in vitro androgenesis in tobacco through iron-free media. Biol. Plant. 21, 412-417.

HEBERLE-BORS, E. and REINERT, J. (1979). Androgenesis in isolated pollen cultures of Nicotiana tabacum: dependence upon pollen development. Protoplasma 99, 237-245.

HEBERLE-BORS, E. (1980). Interaction of activated charcoal and iron chelates in anther cultures of Nicotiana and Atropa belladonna. Z. Pflanzenphysiol 99, 339-347.

HEBERLE-BORS, E. and REINERT, J. (1980). Isolated pollen cultures and pollen dimorphism. Naturwissenschaften 67, 311-312.

HEBERLE-BORS, E. and REINERT, J. (1981). Environmental control and evidence for predetermination of pollen embryogenesis in Nicotiana tabacum pollen. Protoplasma 109, 249-255.

HEBERLE-BORS, E. (1982). In vitro pollen embryogenesis in Nicotiana tabacum L. and its relation to pollen sterility, sex balance, and floral induction of the pollen donor plants. Planta 156, 396-401.

HEBERLE-BORS, E. (1983). Induction of embryogenic pollen grains in situ and subsequent in vitro pollen embryogenesis in Nicotiana tabacum by treatments of the pollen donor plants with feminizing agents. Physiol. Plant. 59, 67-72.

HEBERLE-BORS, E. (1984). Genotypic control of pollen plant formation of Nicotiana tabacum. L. Theor. Appl. Genet. 68, 475-479.

HEBERLE-BORS, E. and ODENBACH, W. (1984). In vitro pollen embryogenesis and cytoplasmic sterility in Triticum aestivum Pflanzenzucht (in press).

HENTRICH, W. and BEGER, B. (1974). Studies on the mutagenous efficiency of N-nitrose-N-methyl urea with Saintpaulia ionantha H. Wendl. Archiv. Zuchtungforsch 4, 29-43.

HERKLOTZ, A. (1964). Influence of constant and daily alternating temperatures on growth and development of Saintpaulia ionantha Wendl. Gartenbauwiss. 29, 424-438.

HIDAKA, T., YAMADA, Y. and SCHICHIJO, T. (1982). Plantlet formation by anther culture of Citrus aurantium L. Japan. J. Breed. 32,247-252.

HIDAKA, T. (1984). Effects of sucrose concentration, pH of media, and culture temperature on anther culture of citrus Japan. J. Breed 34, 416-422.

HILDRUM and KRISTOFFERSEN (1969). The effect of temperature and light intensity on flowering in Saintpaulia ionantha Wendl. Acta Hort. 14,249-255.

HILLMAN, W.S. (1956). Injury of tomato plants by continuous light and unfavourable photoperiodic cycles. Ann. J. Bot. 43, 89-96.

HOLDGATE, D.P. (1977). Propagation of ornamentals by tissue culture. In: Reinhert, J. and Bajaj, Y.P.S. (eds.). Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture. Springer-Verlag, Berlin pp. 18-43.

HORNER, M. and STREET, H.E. (1978). Pollen dimorphism-origin and significance in pollen plant formation by anther culture. Ann Bot. 42, 763-771.

HUANG, B. and SUNDERLAND, N. (1982). Temperature-stress pretreatment in barley anther culture. Ann. Bot. 49, 77-88.

HUGHES, K.W., BELL, S.L. and CAPONETTI, J.D. (1975). Anther-derived haploids of the African violet. Can. J. Bot. 53, 1442-1444.

HUGHES, K.W. (1977). Isolation of protoplasts from haploid plants of the African violet. ASB Bulletin 24, 60.

INSTITUTE OF GENETICS (401 RESEARCH GROUP). ACADEMIA SINICA, PEKING. (1975). Primary study on induction of pollen plants of Zea mays. Acta. Genet. Sin. 2, 138-143.

IMAMURA, J. and HARADA, H. (1980). Stimulatory effects of reduced atmospheric pressure on pollen embryogenesis. Naturwissenschaften. 67, 357-358.

IYER, R.D. and RAINA, S.K. (1972). Planta 104, 146-156.

JACOB, M., DENEE, G. and COUMANS, M. (1980). La production du Saintpaulia ionantha in vitro: aspects economiques. Med. Fac. Landbouww. Rijksuniv. Gent. 45, 335-343.

JIALIN, W., LANQIU, Z., FANGHONG, N., MANLING, C., HUIYING, Z. and BILAN, Z. (1983). Selection of pure line of maize (Zea mays) by anther culture and observations on its hybrids. Sci. Sinica. 26, 725-733.

JOHANSSON, L. and ERIKSSON, T. (1977). Induced embryo formation in anther cultures of several Anemone species. Physiol. Plant. 40, 172-174.

JOHANSSON, L., ANDERSSON, B. and ERIKSSON, T. (1982). Improvement of anther culture technique: Activated charcoal bound in agar medium in combination with liquid medium and elevated CO_2 concentration Physiol. Plant. 54,24-30.

JOHANSSON, L. (1983). Effects of activated charcoal in anther cultures. Physiol. Plant. 59, 397-403.

JOHANSSON, L. and ERIKSSON, T. (1984). Effects of carbon dioxide in anther cultures. Physiol. Plant. 60, 26-30.

JUNGNICKEL, F. (1977). Induktion und vermehrung von mutanten bei Saintpaulia ionantha H. WENDL in sterilkultur. Biol. Zbl. 96, 335-343.

KAMEYA, T. and HINATA, K. (1970). Induction of haploid plants from pollen grains of Brassica. Jpn. J. Breed. 20, 82-87.

KASHA, K.J. (1974) ed. Haploids in Higher Plants, Advances and Potential. University of Guelph, Guelph, Canada.

KASPERBAUER, M.J. and COLLINS, G.B. (1972). Reconstitution of diploids from leaf tissue of anther-derived haploids in tobacco. Crop Sci. 12, 98-101.

KAWAI, T. (1967). New crop varieties by mutation breeding. Jpn. Agric. Res. Quart. 2, 8-12.

KELLER, W.A., RAJHATHY, T. and LACAPRA, J. (1975). In vitro production of plants from pollen in Brassica campestris. Can. J. Genet. Cytol. 17, 655-666.

KELLER, W.A. and ARMSTRONG, K.C. (1977). Embryogenesis and plant regeneration in Brassica napus anther cultures. Can. J. Bot. 55, 1383-1388.

KELLER, W.A. and STRINGHAM, G.R. (1978). Production and utilli-zation of microspore-derived haploid plants. In: Frontiers of Plant Tissue Culture. T.A. Thorpe (eds.) The International Association for Plant Tissue Culture, Calgary.

KELLER, W.A. and ARMSTRONG, K.C. (1979). Stimulation of embryo-genesis and haploid production in Brassica campestris anther cultures by elevated temperature treatments. Theor. Appl. Genet. 55, 65-76.

KELLER, W.A. and ARMSTRONG, K.C. (1981). Production of anther-derived dihaploid plants in autotetraploid marrowstem kale (Brassica oleracea var. acephala). Can. J. Genet. Cytol. 23, 259-265.

KELLY, J.W. and LINEBERGER, R.D. (1981). Thermal neutron induced changes in Saintpaulia Envir. Exp. Bot. 21, 95-102.

KETELLAPPER, H.J. (1960). Interaction of endogenous and environmental periods in plant growth. Plant. Physiol. 35, 238-241.

KHOKHAR, J.A., HUMPHREYS, J.M., SHORT, K.C. and GROUT, B.W.W. (1982) Anthocyanins in African violet. Hortscience 17, 810-811.

KHOKHAR, J.A. (1983). Studies on clonal Propagation and transplanting physiology of African violet. M. Phil thesis. North East London Polytechnic. London.

KHVOSTOVA, V.V. (1967). Mutagenic treatment of agricultural plants. Erwin Baur. Ged. Vorl. 4, 21-27.

KING, G.S. (1949). Direct and transmitted X-ray effects on growth of tobacco callus in vitro. Ann. J. Bot. 36, 265-270.

KIVI, E.I., REKUNEN, M. and VARIS, E. (1974). Use of induced mutations in solving problem of northern crop production. Polyploidy and Induced Mutations in Plant Breeding, pp 187-194, IAEA, Vienna.

KNAUSS, J.F. and MILLER, J.W. (1974). Bacterial blight of Saintpaulia ionantha caused by Erwinia chrysanthemi. Phytopathology 64, 1046-1047.

KONAR, R.N. (1963). A haploid tissue from the pollen of Ephedra foliata Boiss. Phytomorphology. 15, 245-248.

KRUEGER-LEBUS, S., POTRYKUS, I. and IMAMURA, J. (1983). Lycopersicon esculentum: Globular embryos from microspores and calli from diploid protoplasts. In: pp 46-47.

KUKULCZANKA, K. and SUSZYNSKA, G. (1972). Regenerative properties of Saintpaulia ionantha Wendl. leaves cultured in vitro. Acta. Soc. Bot. Pol. 41, 503-509.

LAPINS, K. (1962). Proc. Amer. Soc. Hort. Sci. 81, 26.

LARKIN, P.J. and SCOWCROFT, W.R. (1981). Somaclonal variation a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60, 197-214.

LE, C.L. and COLLET, G.F. (1981). Advantages et contraintes de la multiplication in vitro de Saintpaulia ionantha Wendl. Revue suisse Vitic. Arboric. Hortic. 13, 265-270.

LEENHOUTS, H.P., SIJSMA, M.J., LITWINISZYN, M. and CHADWICK, K.H. (1981). The repair of sub-lethal damage and the stimulated repair of potentially lethal damage in Saintpaulia. Int. J. Radiat. Biol. 40, 413-425.

LEENHOUTS, H.P., BROERTJES, C., SIJSMA, M.J. and CHADWICK, K.H. (1982). Radiation stimulated repair in Saintpaulia: its cellular basis and effect on mutation frequency. Enviro. Exp. Bot. 22, 301-306.

LICHTER, R. (1981). Anther culture of Brassica napus in a liquid culture medium. Z. Pflanzenphysiol 103, 229-237.

LIM-HO, C.L. and LEE, S.M. (1982). Micropropagation of Saintpaulia at Singapore Botanic Gardens Gard. Bull. Singapore 35, 73-81.

LINSMAIER, E.M. and SKOOG, F. (1965). Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18, 100-127.

MAHESHWARI, S.C., TYAGI, A.K. and MALHOTRA, K. (1980). Induction of haploidy from pollen grains in angiosperms - the current status. Theor. Appl. Genet. 58, 193-206.

MAHESHWARI, S.C., RASHID, A. and TYAGI, A.K. (1982). Haploids from pollen grains - retrospect and prospect. Amer. J. Bot. 69, 865-879.

MALIGA, P. (1980). Isolation, characterization, and utilization of mutant cell lines in higher plants. In: I.K. Vasil (ed.), Perspectives in plant cell and tissue culture, pp 225-250. Int. Rev. Cytol. (Suppl.) 11A. Academic Press, New York.

MARKS, G.E. (1973). A rapid HCl/Toluidine blue squash technic for plant chromosomes Stain. Technol. 48, 229-231.

MARSOLAIS, A.A., SEGUIN-SWARTZ, G. and KASHA, K.J. (1984). The influence of anther cold pretreatments and donor plant genotypes on in vitro androgenesis in wheat (Triticum aestivum L.). Plant. Cell Tissue Organ Culture 3, 69-79.

MILLER, C.O. (1963). Kinetin and Kinetin like compounds, pp. 194-202, In: Moderne Methoden der Pflanzenanalyse, vol. VI eds K. Paech & M.V. Tracey Springer-Verlag, Berlin.

MOKHTARZADEH, A. and CONSTANTIN, M.J. (1978). Plant regeneration from hypocotyl- and anther-derived callus of berseem clover. Crop. Sci. 18, 567-572.

MONCOUSIN, C. (1978). Contribution a la mise au point d'un milieu de culture pour la multiplication in vitro de Gesneriacees Rev. Hortic. Suisse 51, 295-301.

DE MORAES-FERNANDES, M.I.B. and PICARD, E. (1983). Viability of haploid production by anther culture using brazilian wheat genotypes. Rev. Brasil. Genet. 6, 261-277.

MURASHIGE, T. and SKOOG, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15, 473-497.

MURASHIGE, T. and NAKANO, R. (1966). Tissue culture as a potential tool in obtaining polyploid plants. J. Hered. 57, 115-118.

NABORS, M., GIBBS, S.E., BERNSTEIN, C.S. and MEIS, M.E. (1980). NaCl-tolerant tobacco plants from cultured cells. Z. Pflanzenphysiol 97, 13-17.

NAGMANI, R. and RAGHAVAN, V. (1983). Induction of embryogenic divisions in isolated pollen grains of Hyoscyamus niger in a single-step method. Z. Pflanzenphysiol 109, 87-90.

NAKAMURA, A. and ITAGAKI, R. (1973). Anther culture in Nicotiana and the characteristics of the haploids. Jpn. J. Breed 23, 71-78.

NAYLOR, E.E. and JOHNSON, B. (1937). A histological study of vegetative reproduction in Saintpaulia ionantha. Am. J. Bot. 24, 673-678.

NEILSON-JONES, W. (1969). Plant Chimeras, 2nd Ed. Methuen, London.

NIIZEKI, H. and OONO, K. (1968). Induction of haploid rice plants from anther culture. Proc. Jpn. Acad. 44, 554-557.

NIIZEKI, H. and OONO, K. (1971). Rice plants obtained by anther culture. In: Les Cultures de Tissus des Plantes. Colloq. Int. CNRS (Paris). 193, 251-257.

NITSCH, J.P. (1969). Experimental androgenesis in Nicotiana. Phytomorphology 19, 389-404.

NITSCH, J.P. and NITSCH, C. (1969). Haploid plants from pollen grains. Science 163, 85-87.

NITSCH, J.P. (1971). Les plantes sans meres. La Recherche 2, 523-529.

NITSCH, J.P. (1972). Haploid plants from pollen. Z. Pflanzenzucht 67, 3-18.

NITSCH, C. and NORREEL, B. (1972). Factors favouring the formation of androgenetic embryos and homozygous plants. pp 129-144. In: A.M. Srb. (ed). Genes, Enzymes and Populations. Plenum, New York.

NITSCH, C. and NORREEL, B. (1973). Effect d'un choc thermique sur le pouvoir embryogene du pollen de Datura innoxia cultive dans l'anthere ou isole de l'anthere. C.R. Acad. Sci. 276, 303-306.

NITSCH, C. (1974). Pollen culture- a new technique for mass production of haploid and homozygous plants. In: K.J. Kasha (ed.), Haploids in higher plants - advances and potential, pp 123-135. University of Guelph, Guelph.

NITSCH, C. (1975). Single cell culture of a haploid cell: The microspore. In Ledoux, L. (Ed.): Genetic Manipulations with Plant Material, pp. 297-310. London: Plenum Press.

NITSCH, C. (1977). Culture of isolated microspores. In: J. Reinert and Bajaj, Y.P.S. (eds.), Plant, cell, tissue and organ culture, pp. 268-278, Springer-Verlag, Berlin.

NORRIS, R.E., SMITH, R.H. and TURNER, P. (1982). Phenotypic differences in haploid African violets. In Vitro. 18, 443-446.

OUYANG, T.-W., HU, H., CHUANG, C.-C. and TSENG, C.-C. (1973). Induction of pollen plants from anthers of Triticum aestivum L. cultured in vitro. Sci. Sinica. 16, 79-95.

ONO, K. and HARASHIMA, S. (1981). Induction of haploid callus from isolated microspores of peony in vitro. Plant Cell. Physiol. 22, 337-341.

ONO, K. and HARASHIMA, S. (1983).

OONO, K. (1981). In vitro methods applied to rice. In: Plant Tissue Culture. Methods and Applications in Agriculture (T.A. Thorpe, ed) pp. 273-298. Academic Press, New York.

OSWALD, T.H., SMITH, A.E. and PHILLIPS, D.V. (1978). Phytotoxicity and detoxification of metribuzin in dark-grown suspension cultures of soybeans. Pestic. Biochem. Physiol. 8, 73-83.

PELLETIER, G. and ILAMI, M. (1972). Les facteurs de l'androgenese in vitro chez Nicotiana tabacum. Z. Pflanzenphysiol 68, 97-114.

PELLETIER, G. (1973). Les conditions et les premiers stades de l'androgenese in vitro chez Nicotiana tabacum. Mem. Soc. Botan. Fr. Colloq. Morphologie 261-268.

PELLETIER, G. and HENRY, Y. (1974). Cold pretreating flower buds of Nicotiana tabacum L. Haploid Information Service 10, 5-8.

PICARD, E. (1973). Influence de modifications dans les correlations internes sur le devenir du gametophyte male de Triticum aestivum L. in situ et en culture in vitro. C.R. Acad. Sci. 277D, 777-780.

PICARD, E. and DE BUYSER, J. (1975). Nouveaux resultats concernant la culture d'anthere in vitro de ble tendre. (Triticum aestivum L.). Effects d'un choc thermique et de la position de l'anthere dans l'epi. C.R. Acad. Sci. Ser. D. 281, 127-130.

PICARD, E. and DE BUYSER, J. (1977). Ann. Ameloir. Plant 27, 483-488.

PLATE, H.P. and KROBER, H. (1972). Eine neue Phytophthora-krankheit an Saintpaulien Gartenwelt 24, 513-516.

POHLHEIM, F. and BEGER, B. (1974). Erhohung der mutationstrate im plastom bei Saintpaulia durch N-Nitroso-N-Methylharnstoff. Biol. Rdsch. 12, 204-6.

PREIL, W., ENGELHARDT, M. and WALTHER, F. (1983). Breeding of low temperature tolerant Poinsettia (Euphorbia pulcherrima) and Chrysanthemum by means of mutation induction in vitro culture. Acta. Hort. 131, 345-351.

PRIMO-MILLO, E. and SUNDERLAND, N. (1976). Effect of plant-growth-temperature on pollen embryogenesis in Nicotiana Knightiana. John Innes Inst. Annu Rep 66, 233-235.

PROTOPLASTS 1983. Poster Proceedings, 6th International Protoplast Symposium, Basel, August 12-16, 1983, Birkhauser Verlag, Basel-Boston - Stuttgart. 46-47.

RAGHAVAN, V. (1976). Role of the generative cell in androgenesis in henbane. Science 191, 388-389.

RAGHAVAN, V. (1978). Origin and development of pollen embryoids and pollen calluses in cultured anther segments of Hyoscyamus niger (henbane). Amer. J. Bot. 65, 948-1002.

RAINA, S.K. and IYER, R.D. (1973). Differentiation of diploid plants from pollen callus in anther cultures of Solanum melongena L. Z. Pflanzenzucht. 70, 275-280.

RAQUIN, C. and PILET, V. (1972). Production de plantules a partir d'antheres de Petunias cultivees in vitro. Compt Rend. D274, 1019-1022.

RAQUIN, C. (1982). Analysis of segregations of genes involved in floral pigmentation of Petunias obtained from androgenesis. C.R. Acad. Sc. Paris (Series III) 294, 335-338.

RASHID, A. and REINERT, J. (1980). Selection of embryogenic pollen from cold-treated buds of Nicotiana tabacum var. Badischer Burley and their development into embryos in cultures. Protoplasma. 105, 161-167.

RASHID, A. and REINERT, J. (1981a). Differentiation of embryogenic pollen in cold-treated buds of Nicotiana tabacum var. Badischer Burley and nutritional requirements of the isolated pollen to form embryos. Protoplasma 106, 137-144.

RASHID, A. and REINERT, J. (1981b). High-frequency embryogenesis in ab initio pollen cultures of Nicotiana tabacum. Naturwissenschaften 68, 378-379.

RASHID, A. (1982). Induction of embryos in pollen cultures of Nicotiana sylvestris. Physiol. Plant. 56, 223-224.

RASHID, A. (1983). Induction of embryos in ab initio pollen cultures of Nicotiana. In Plant Cell Culture in Crop Improvement. 22, pp 141-144 Eds Sen & Giles, Plenum Press.

REED, S.C. (1954). African violet genetics. J. Hered. 45, 225-230.

REINERT, J., BAJAJ, Y.P.S. and HEBERLE, E. (1975). Induction of haploid tobacco plants from isolated pollen. Protoplasma 84, 191-196.

REINERT, J. and BAJAJ, Y.P.S. (1977). Anther culture: haploid production and its significance. In: J. Reinert and Y.P.S. Bajaj (eds.), Plant cell tissue, and organ culture pp. 251-267.

ROBERTS, A.V. and SHORT, K.C. (1979). An experimental study of mitosis. J. Biol. Ed. 13, 195-198.

ROGOZINSKA, J.H. and GOSKA, M. (1982). Attempts to induce haploids in anther cultures of sugar, fodder and wild species of beet. Acta. Soc. Bot. Pol. 51, 91-105.

SANGWAN, R.S. and NORREEL, B. (1975). Induction of plants from pollen grains of Petunia cultured in vitro. Nature 257, 222-224.

SANGWAN-NORREEL, B.S. (1977). Androgenic stimulating factors in the anther and isolated pollen grain culture of Datura innoxia Mill. J. Exp. Bot. 28, 843-852.

SCHAEFFER, G.W., SHARPE, F.T. and CREGAN, P.B. (1985). Variation for improved protein and yield from rice anther culture. Theor. Appli. Gen. 67, 383-388.

SCHLEGEL, G. (1982). Influence of low temperature on regeneration of Saintpaulia ionantha H. Wendl. In: Abstract 21st Intn. Hort. Congress Hamburg. Intn. Soc. Hort. Sci. vol.2 Abstract No. 1719.

SHARP, W.R., DOUGALL, D.K. and PADDOCK, E.F. (1971). Haploid plantlets and callus from immature pollen grains of Nicotiana and Lycopersicon. Bull. Torrey Botan. Club 98, 219-222.

SHARP, W.R. and RASKIN, R.S. (1972). The use of nurse culture in the development of haploid clones in tomato. Planta. 104, 357-361.

SHARP, W.R., RASKIN, R.S. and SOMMER, H.E. (1972). Haploidy in Lilium. Phytomorph. 21, 334-337.

SHARP, W.R., CALDAS, L.S. and CROCOMO, O.J. (1973). Studies on the induction of Coffea arabica callus from both somatic and microsporogenous tissues; and subsequent embryoid and plantlet formation. Am. J. Botany 60(4), 13.

SHIMADA, T. and MAKINO, T. (1975). In vitro culture of wheat. III. Anther culture of the A genome aneuploids in common wheat. Theor. Appl. Genet. 46, 407-410.

SITBON, (1973). In: La culture d'antheres in vitro, C. Dore and A. Lambert. Revue Hort (Paris) 145, 44-48.

SKIRVIN, R.M. and JANICK, J. (1976). Tissue culture-induced variation in scented Pelargonium spp. J. Am. Soc. Hort. Sic. 101, 281-290.

SKIRVIN, R.M. (1978). Natural and induced variation in tissue culture. Euphytica 27, 241-266.

SMITH, R.H., KAMP, M. and DAVIES, R.S. (1981). Reduced plant size of haploid African violets. In vitro. 17, 385-387.

SOPORY, S.K. and MAHESHWARI, S.C. (1976). Development of pollen embryoids in anther cultures of Datura innoxia. I. General observations and effects of physical factors. J. Exp. Bot. 27, 49-57.

SOPORY, S.K. JACOBSEN, E. and WENZEL, G. (1978). Production of monohaploid embryoids and plantlets in cultured anthers of Solanum tuberosum. Plant. Sci. Lett. 12, 47-54.

SOPORY, S.K. (1979). Effect of sucrose, hormones, and metabolic inhibitors on the development of pollen embryoids in anther cultures of dihaploid Solanum tuberosum. Can. J. Bot. 57, 2691-2694.

SPARROW, H., SPARROW, R.C. and SCHAIRER, L.A. (1960). The use of X-rays to induce somatic mutations in Saintpaulia. African Violet Mag. 13, 32-37.

START, N.D. and CUMMING, B.G. (1976). In vitro propagation of Saintpaulia ionantha Wendl. Hortscience 11, 204-206.

SUGIURA, T. (1936). Studies on the chromosome numbers in Higher plants with special reference to cytokinesis, 1. Cytologia. 7, 544-595.

SUKAMARAN, N.P. and WEISER, C.J. (1972). An excised leaflet test for evaluating potato frost tolerance. Hortscience 7, 467-468.

SUNDERLAND, N. and WICKS, F.M. (1969). Cultivation of haploid plants from tobacco pollen. Nature 224, 1227-1229.

SUNDERLAND, N. (1971). Anther culture: a progress report. Sci. Prog. 59, 527-549.

SUNDERLAND, N. and DUNWELL, J.M. (1971). John Innes Ann. Rept. no. 62, pp. 60-61.

SUNDERLAND, N. and WICKS, F.M. (1971). Embryoid formation in pollen grains of Nicotiana tabacum. J. Exp. Bot. 22, 213-26.

SUNDERALND, N. (1974). Anther culture as a means of haploid induction. In: Haploids in Higher Plants: Advances and Potential (K.J. Kasha, ed.) pp. 91-122. Guelph Univ. Press, Guelph.

SUNDERLAND, N. and DUNWELL, J.M. (1974). Pathways in pollen embryogenesis, pp. 141-167. In: Tissue Culture and Plant Science, 1974 (H.E. Street, ed) pp. 141-167. Academic Press, New York.

SUNDERLAND, N., COLLINS, G.B. and DUNWELL, J.M. (1974). The role of nuclear fusion in pollen embryogenesis of Datura innoxia Mill. Planta 117, 227-241.

SUNDERLAND, N. (1977). Nuclear cytology. In: Plant Tissue and Cell Culture (H.E. Street, ed.) pp. 177-205. Univ. California Press, Berkeley.

SUNDERLAND, N. and DUNWELL, J.M. (1977). Anther and pollen culture. In: Plant Tissue and Cell Culture (Ed. H.E. Street), Blackwell Scientific Publications: Oxford, England, 223-265.

SUNDERLAND, N. and ROBERTS, M. (1977). New approach to pollen culture. Nature 270, 236-238.

SUNDERLAND, N. (1978). Strategies in the improvement of yields in anther culture. In: Proceedings of symposium on plant tissue culture, pp. 65-86. Science Press, Peking.

SUNDERLAND, N. and ROBERTS, M. (1979). Cold-pretreatment of excised flower buds in float culture of tobacco anthers. Ann. Bot. 43, 405-414.

SUNDERLAND, N. and WILDON, D.C. (1979). A note on the pretreatment of excised flower buds in float culture of Hyoscyamus anthers. Plant. Sci. Lett. 15, 169-175.

SUNDERLAND, N. (1980). Guidelines in the culture of pollen in vitro pp 33-40. In: Tissue Culture Methods for Plant Pathologists, ed. Ingram, D.S. and Helgeson. J.P.

SUTTER, E. and LANGHANS, R.W. (1981). Abnormalities in Chrysanthemum regenerated from long term cultures. Ann. Bot. 48, 559-568.

TAL, Vol III.

TAGUCHI, T. and MII, M. (1982). Effects of chilling, anther preculture and growth regulators on embryogenesis in isolated pollen culture of Nicotiana rustica L. Japan J. Breed 32, 303-310.

TAKAYAMA, S. and MISAWA, M. (1982). Mass propagation of ornamental plants through tissue culture. Proc. 5th Intl. Cong. Plant Tissue and Cell Culture. pp 681-682.

TAN, B.H. and HALLORAN, G.M. (1982). Pollen dimorphism and the frequency of inductive anthers in anther cultures of Triticum monococcum. Biochem. Physiol. Pflanz. 177, 197-203.

TEMPLETON-SOMERS, K.M., SHARP, W.R. and PFISTER, R.M. (1981). Selection of cold-resistant cell lines of carrot. Z. Pflanzenphysiol 103, 139-148.

THANH-TUYEN, N.T. and deGUZMEN, E.V. (1983). Formation of pollen embryos in cultured anthers of coconut (Cocus nucifera L.) Plant. Sci. Lett. 29, 81-88.

THEILER-HEDRICH, R. and THEILER-HEDRICH, C.M. (1983). The use of tissue culture for the production of ornamental plants: some economic aspects. Acta. Hort. 131, 179-192.

THURLING, N. and CHAY, P. (1984). The influence of donor plant genotype and environment on production of multicellular microspores in cultured anthers of Brassica napus ssp. oleifera. Ann. Bot. 54, 681-693.

TYAGI, A.K., RASHID, A. and MAHESHWARI, S.C. (1981). Promotive effect of polyvinylpolypyrrolidone on pollen embryogenesis in Datura innoxia Mill. Physiol Plant 53, 405-406.

TYAGI, A.K., RASHID, A, and MAHESHWARI, S.C. (1981). Sodium chloride resistant cell line from haploid Datura innoxia Mill. - a resistant trait carried from cell to plantlet and vice versa in vitro. Protoplasma 105, 327-332.

THOMAS, E. and WENZEL, G. (1975b). Embryogenesis from microspores of Brassica napus. Z. Pflanzenzuecht. 74, 77-81.

THURLING, N.T. and CHAY, P.M. (1984). The influence of donor plant genotype and environment on production of multicellular microspores in cultured anthers of Brassica napus ssp oleifera. Ann. Bot. 54, 681-693.

TULECKE, W. (1953). A tissue derived from the pollen of Ginkgo biloba. Science. 117, 599-600.

TULECKE, W. (1957). The pollen of Ginkgo biloba: in vitro culture and tissue formation. Am. J. Bot. 44, 602-608.

TULECKE, W. (1959). The pollen cultures of C.D. Larue: a tissue from the pollen of Taxus. Bull. Torrey Botan. Club. 86, 283-289.

TULECKE, W. and SEHGAL, N. (1963). Cell proliferation from pollen of Torreya nucifera. Contrib. Boyce Thompson Inst. 22, 153-163.

TYAGI, A.K., RASHID, A. and MAHESHWARI, S.C. (1979). High frequency production of embryos in Datura innoxia from isolated pollen grains by combined cold treatment and serial culture of anthers in liquid medium. Protoplasma 99, 11-17.

TYAGI, A.K., RASHID, A. and MAHESHWARI, S.C. (1981). Promotive effect of polyvinylpolypyrrolidone on pollen embryogenesis in Datura innoxia Mill. Physiol. Plant 53, 405-406.

TYAGI, A.K., RASHID, A. and MAHESHWARI, S.C. (1981). Sodium chloride resistant cell line from haploid Datura innoxia Mill. - a resistant trait carried from cell to plantlet and vice versa in vitro. Protoplasma 105, 327-332.

VAGERA, J. and HAVRANEK, P. (1982). In vitro regulation of androgenesis by iron ions and chelate: A common property of two androgenic species (Nicotiana tabacum L. and Datura innoxia MILL.) Biol. Plant. 24, 282-289.

VAGERA, J. and JILEK, M. (1984). Specification of the effect of chelating complex of iron ions in androgenesis in vitro by means of cation-free minimal medium. Biol. Plant. 26, 121-127.

VALLEJOS, C.E. (1979). Genetic diversity of plants for response to low temperature and its potential use in crop plants. In: Lyons, J.M., Graham, D. and Raison, J.K. (eds). Low Temperature Stress in Crop Plants, New York, Academic Press. pp. 473-489.

VAN ARKEL, H. (1977). New forage crop introductions for the semi-arid highland areas of Kenya as a means to increase beef production. Netherlands. J. Agric. Sci. 25, 135-150.

VASIL, I.K. (1967).
Biol. Rev. Cambridge Philos. Soc. 42, 327-373.

VASIL, I.K. (1973).
Naturwissenschaften 60, 247-253.

VASIL, I.K. (1974). In: "Fertilization in Higher Plants" (H.F. Linskens, ed.), pp 105-118. North-Holland Publ., Amsterdam.

VASIL, I.K. (1980). Androgenetic haploids Int. Rev. Cyto. Supp. 11A, 195-223.

VAZQUEZ, A.M., DAVEY, M.R. and SHORT, K.C. (1977). Organogenesis in cultures of Saintpaulia ionantha Acta. Hort. 78, 249-259.

VAZQUEZ, A.M. and SHORT, K.C. (1978). Morphogenesis in cultured floral parts of African violet. J. Exp. Bot. 29, 1265-1271.

WAGNER, G. and HESS, D. (1974). Haploide, diploide and triploide pflanzen von Petunia hybrida aus pollenkorner. Z. Pflanzenphysiol. 73, 273-276.

WAKASA, K. (1979). Variation in the plants differentiated from tissue culture of pineapple. Jpn. J. Breed. 29, 13-22.

WALTHER, F. and PREIL, W. (1981). Mutants tolerant to low-temperature conditions induced in suspension culture as a source for improvement of Euphorbia pulcherrima. Wild. ex klotzsch. In: Induced mutations - a tool in plant breeding IAEA. Vienna: 399-405.

WANG, Y.Y., SUN, C.-S., WANG, C.-C. and CHIEN, N.-F. (1973). The induction of pollen plantlets of Triticale and Capsicum annuum from anther culture. Sci. Sinica 16, 147-151.

WANG, C.C., SUN, C.S. and CHU, C.C. (1974). On the condition for the induction of rice pollen plantlets and certain factors affecting the frequency of induction. Acta. Bot. Sinica 16, 43-53.

WARFIELD, D. (1973). Induction of mutations in African violet (Saintpaulia ionantha Wendl.) by Ethyl methanesulfonate. Hortscience 8, 29.

WARFIELD, D.L., NILAN, R.A. and WITTERS, R.E. (1975). The effect of ethylene and ionizing radiation on Saintpaulia peroxidase activity. Radiat. Bot. 15, 423-429.

WANTANABE, K., NISHI, Y. and TANAKA, R. (1972). Anatomical observations on the high frequency callus formation from anther culture of Chrysanthemum. Jpn. J. Genet. 47, 249-255.

WEATHERHEAD, M.A., BURDON, J. and HENSHAW, G.G. (1978). Some effects of activated charcoal as an additive to plant tissue culture media. Z. Pflanzenphysiol. 89, 141-147.

WEATHERHEAD, M.A. and HENSHAW, G.G. (1979). The induction of embryoids in free pollen culture of potatoes. Z. Pflanzenphysiol. 94, 441-447.

WEATHERHEAD, M.A., GROUT, B.W.W. and SHORT, K.C. (1982). Increased haploid production in Saintpaulia ionantha by anther culture. Scientia Hortic. 17, 137-144.

WENDLAND, H. (1983). Saintpaulia ionantha. Gartenflora 40, 321-323.

WENT, F.W. (1959). The periodic aspect of photoperiodism and thermoperiodicity. In: Photoperiodism and Related Phenomena pp. 551-564. American Association for the Advancement of Science. Washington, D.C.

WENT, F.W. (1960). Photo- and thermoperiodic effects in plant growth. Cold Spring Harbor Symposia in Quantitative Biology 25, 221-230. Long Island Biological Association. Cold Spring Harbor. New York.

WENT, F.W. (1962). Ecological implications of the autonomous 24-hour rhythm in plants. Ann. New York Acad. Sci. 98, 866-875.

WENZEL, G. and THOMAS, E. (1974). Observations on the growth in culture of anthers of Secale cereale. Z. Pflanzenzucht. 72, 89-94.

WENZEL, G., HOFFMANN, F., POTRYKUS, I. and THOMAS, E. (1975). The separation of viable rye microspores from mixed populations and their development in culture. Molec. Gen. Genet. 138, 293-297.

WENZEL, G., HOFFMAN, F. and THOMAS, E. (1977). Increased induction and chromosome doubling of androgenic haploid rye. Theor. Appl. Genet. 51, 81-86.

WERNICKE, W. and KOHLENBACH, H.W. (1975). Anther cultures in the genus Scopolia. Z. Pflanzenphysiol. 77, 89-93.

WERNICKE, W. and KOHLENBACH, H.W. (1976). Investigations on liquid culture medium as a means of anther culture in Nicotiana. Z. Pflanzenphysiol. 79, 189-198.

WERNICKE, W.C. and KOHLENBACH, H.W. (1977). Experiments on the culture of isolated microspores in Nicotiana and Hyoscyamus. Z. Pflanzenphysiol. 81, 330-340.

WERNICKE, W., HARMS, C.T., LORZ, H. and THOMAS, E. (1978). Selective enrichment of embryogenic microspore populations. Naturwissenschaften 65, 540-541.

WIDHOLM, J. (1978). The selection of agriculturally desirable traits with cultured plant cells. In: Hughes, K.W., Henke, R. and Constantin, M. (Eds): Propagation of Higher Plants Through Tissue Culture, Technical Information Center. Untied States Department of Energy, Knoxville.

WILSON, G.B. (1951). A note on the cytology of Saintpaulias. African Violet Mag. 5, 18-19.

WILSON, H.M. (1977). Culture of whole barley spikes stimulates high frequencies of pollen calluses in individual anthers. Plant. Sci. Lett. 9, 233-238.

WILSON, H.M., MIX, G. and FOROUGH-WEHR, B. (1978). Early microspore divisions and subsequent formation of microspore calluses at high frequency in anthers of Hordeum vulgare L. J. Exp. Bot. 29, 227-238.

WONG, C.-K., KO, S.-W. and WOO, S.-C. (1983). Regeneration of rice plantlets on NaCl-stressed medium by anther culture. Bot. Bull. Acad. Sin. 24, 59-64.

XU, L.-Q. (1984). The promotive effects of ginseng on rapid clonal in vitro propagation of African violet (Saintpaulia ionantha Wendl.) Acta. Bot. Sin. 26, 489-498.

YAMADA, T., SHOJI, T. and SINOTO, Y. (1963). Formation of calli and free cells in tissue culture of Tradescantia reflexa. Bot. Mag. 76, 332-339.

ZENKTELER, M., MISIURA, E. and PONITKA, A. (1975). Induction of androgenetic embryoids in the in vitro cultured anthers of several species. Experientia 31, 289-291.

ZENKTELER, M. and STEFANIAK, B. (1982). Induction of androgenesis in anthers of Hordeum vulgare L. cultured in vitro on leaves and calluses. Plant. Sci. Lett. 26, 219-225.

ZHOU, J.Y. (1980). Pollen dimorphism and its relation to formation of pollen embryos in anther culture of wheat Triticum aestivum. Acta. Bot. Sinica 22, 117-122.

Advanced Studies undertaken in connection with the programme of research

1. Attendance of special lecture/tutorial programme on the following topics

a) vegetative methods for the propagation of ornamental plants.

b) impact of plant tissue culture on horticulture and agriculture.

c) methods for the culture of plant cells and organs *in vitro*

d) culture of pollen and anther tissue and its potential for plant improvement.

e) physiology and metabolism of cultured cells and tissues.

f) effects of temperature on plant growth and development.

g) elimination of systematic pathogens by *in vitro* methods.

h) selection of variant cell lines with enhanced resistance to stress.

i) physiology of tissue cultured plantlets on transfer to soil.

j) analysis of tissue cultured plants for phenotypic and cytological stability.

2. Attendance at short courses offered at Biology Department, North East London Polytechnic

a) aspects of cell technology.

b) cryo preservation of cells and tissues.

3. Meetings attended

a) Society for Experimental Biology, University Leicester, January 1982.

b) International Association of Plant Tissue Culture meeting, Bath 1982.

c) British Plant Growth Regulator Group meeting; Growth regulators in plant sciences, North East London Polytechnic, April 1982.

d) 8th Long Ashton Symposium, Improvement of vegetatively propagated plants, September 1982.

e) The Royal Society of Plant Tissue Culture - Achievement and prospects, May 1983.

f) 41st Easter School University Nottingham Plant Tissue Culture and its Agricultural Implications, September 1984.

INTERNATIONAL SYMPOSIUM PLANT TISSUE AND CELL CULTURE APPLICATION TO CROP IMPROVEMENT OLOMOUC CZECHOSLOVAKIA 24–29 SEPTEMBER 1984

PROCEEDINGS

EDITED BY F. J. NOVÁK, L. HAVEL, J. DOLEŽEL
INSTITUTE OF EXPERIMENTAL BOTANY

CZECHOSLOVAK ACADEMY
OF SCIENCES
PRAGUE 1984

IN VITRO SELECTION FOR COLD-TOLERANT CELL LINES OF *SAINTPAULIA IONANTHA* (WENDL)

J. WARBURTON *, B.W.W. GROUT **, K.C. SHORT *

*Department of Life Sciences, Trent Polytechnic, Nottingham U.K.
** Department of Biology, Plymouth Polytechnic, Plymouth U.K.

Since it has not been possible to obtain single cell cultures of *Saintpaulia*, cultured leaf-disc callus explants, which exhibit precocious adventitious shoot production (of single cell origin), have been used for studies on the selection of cold-tolerant clones.

Previous work (SCHLEGEL, 1982) has shown that explants cultured *in vitro* for 16 weeks showed no regeneration at temperatures below 15°C, although a few clones survived at 12°C. In this study three lines, anther derived (WEATHERHEAD *et al.*, 1982) and diploid cell lines of African Violet (var Blue Rhapsody) and the existing cold-tolerant variety Endurance (BILKEY, 1981) were screened for their ability to tolerate and exhibit morphogenesis at low temperatures. The selection procedure adopted required leaf-disc callus explants to survive a period of six months at temperatures of 8-10°C, then resume growth on return to 25°C. Results indicate that African Violet leaf-disc callus explants can be cultured at 10°C for six months (in the light and dark) with no loss of growth potential as exhibited when the cultures are returned to 25°C. Further incubation at 10°C is detrimental to the diploid but not to the anther-derived lines which can tolerate low temperatures for up to 9 months. The growth rate of the anther-derived Blue Rhapsody callus is more than twice that of the diploid parental callus. Also it more readily undergoes morphogenesis as it produced about 50% more shoots than the parent. Comparison of the above cell lines with those derived from the commercially available cold-tolerant African Violet (var Endurance) revealed that both Endurance and anther-derived cells produced about 20-30% more tissue than the Blue Rhapsody parent. Furthermore, comparison of the morphogenetic potential of the three cell lines, when cultured at 10°C, indicate that Endurance produced about 40% more shoots than Blue Rhapsody. However anther-derived callus produced 8% more shoots than Endurance.

Exposure of plant material to low temperatures often results in the disruption of cell membranes with a consequential release of electrolytes. Thus a measure of the electrolyte leakage from plant material provides a rapid method for indicating the cold tolerance of tissues. Leakage was therefore measured in the three lines being studied. The analysis of electrolyte leakage from isolated leaf-discs incubated at 10°C revealed that both the anther-derived and Endurance lines exhibited about 90% of the leakage found in Blue Rhapsody which indicates that both lines are more tolerant to low temperatures.

A previous report (WARFIELD, 1973) indicated that treating *Saintpaulia* petioles with Ethyl methanesulphonate (EMS) induced 13% mutations in the plantlets produced. Therefore by adopting a similar procedure attempts were made to induce somatic mutations that tolerate

temperatures as low as 8°C. Leaf-discs of anther-derived Blue Rhapsody plants were exposed to 0.25 M EMS for 30 min prior to incubation at 8°C for four months. Control material did not survive this low temperature. The mutagenic treatment resulted in a 30% survival of explants some of which initiated a limited amount of callus. Transfer of this low temperature initiated callus to 25°C resulted in rapid growth and plantlet production. Plants derived from this callus are being evaluated for their ability to tolerate low temperatures.

Plantlets derived from anther initiated callus exhibited approximately 30% more biomass and 50% more adventitious shoot production than the parent Blue Rhapsody when cultured *in vitro* at 10°C for six months. These values were 10% higher than those obtained for the existing cold-tolerant line Endurance. Therefore *in vitro* methods can be expoited for selection of cold-tolerant lines.

References

BILKEY, P. 1981. An assessment of the suitability of *Saintpaulia* for plant genetic manipulations. Ph.D. Thesis, University of Nottingham.

SCHLEGEL, G. 1982. Influence of low temperature on regeneration of *Saintpaulia ionantha* (H. Wendl). Int. Soc. for Hort. Sci. Vol. II, Abs No. 1719.

WARFIELD, D. 1973. Induction of mutations in African Violet *Saintpaulia ionantha* Wendl.) by Ethyl methansulfonate. Hort. Science 8 (1):29.

WEATHERHEAD, M.A., GROUT, B.W.W. and SHORT, K.C. 1983. Increased haploid production in *Saintpaulia ionantha* by anther culture. Scienta. Hort. 17: 137-144.